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DIFFERENTIAL REDUCTION OF METHYLENE BLUE BY CORYMORPHA PALMA[1]

(Twenty-four figures)

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THE data presented in this paper were obtained during the summer of 1934 at the Scripps Institution of Oceanography, La Jolla, California. The authors desire to express their appreciation of the kindness of Dr. T. Wayland Vaughan, director of the Institution, in providing space and facilities for work.

The experiments were undertaken in order to determine whether the gradients indicated by other methods in the various parts of the *Corymorpha* body could be made evident by the reduction of methylene blue in the tissues in low oxygen.

METHODS

It was first necessary to determine, by trial, concentrations of methylene blue and periods of staining which give sufficient color to show differences in rate of reduction in different regions but which are not appreciably toxic and do not bring about appreciable injury or cytolysis during the period of experiment. In some of the experiments animals or isolated parts remained for certain periods in the dye solution open to air and were then sealed under cover glass on depression slides, air bubbles being carefully excluded. In other cases

[1] Assistance in this investigation was provided from a grant by the Rockefeller Foundation in aid of research in the biological sciences at the University of Chicago.

the material was placed in the methylene blue solution and sealed at once. Melted vaseline around the edges of the cover glass served for sealing. For the larger animals or parts tested, large tissue-culture depression slides with depression volume of approximately 2 cc. and for smaller individuals or parts, slides with smaller depression volumes, down to about 0.2 cc., were used. In all cases the depth and volume of the depression was such that the position of the material could be changed in the fluid either by changing position of the slide with respect to gravity or, in the large volumes, by rapidly turning the slide back and forth. Great care was taken to provide for almost continuous change of position of the material in the fluid in order that distribution of oxygen in the fluid might remain as uniform as possible.

In the sealed preparation the animals or parts consume the dissolved oxygen, and reduction of the dye becomes evident sooner or later according to volume of fluid and amount of living material. Most or all parts of the *Corymorpha* ectoderm reduce methylene blue in relatively high oxygen content. Hydranths often show some reduction even in standing water open to the air when 3–4 cm. below the surface. The larger the amount of living material in proportion to volume of fluid in the sealed preparations, the sooner do reduction and decoloration become evident. After the necessary preliminary experiments the proportions of material and fluid were usually so adjusted that reduction of stained material became evident in 10–30 minutes after sealing.

In preparations which are sealed immediately after being placed in the dye solution, concentration of dye can be so adjusted to the material that the parts which reduce more rapidly do not stain at all, that is, they reduce the dye as rapidly as it enters, while regions of less rapid reduction become stained at first and reduce later.

Different parts of the *Corymorpha* body differ greatly in rate of staining and apparently in susceptibility to methylene blue (Child, 1926*a*). With sufficient staining the reducing power of any part may be greatly decreased or completely lost, and further staining leads finally to cytolysis. In attempting to determine differences in rate of reduction, it is necessary to avoid this toxic effect of the dye as far as possible. In experiment this has been done by determining a

range of concentrations and periods of staining preceding reduction which permit reduction in all parts of the material concerned and recovery following opening of the sealed preparation. Then, with increase in concentration or length of staining period, a point is reached at which the reducing power of a certain part is decreased or apparently completely lost. Since the part of an organ or region which begins to reduce first or reduces most rapidly is usually the first to show the decrease or loss of reducing power with continued staining, the appearance of this toxic effect is easily recognizable.

The concentrations of methylene blue used with *Corymorpha* material range from 1/10,000 to 1/500,000. The same reduction gradients can be made evident in most parts of the body with the whole, or almost the whole, range of concentration by varying the staining period preceding reduction.

By opening the sealed preparation after reduction has occurred, it is easily shown that decoloration is due to reduction of the dye in the cells rather than to its loss from the cells. On exposure of the preparation to the air, the color returns rapidly. Moreover, if reduction occurred in the dye solution used for staining, the staining is deeper on reoxidation than before reduction, because more dye has been taken up by the cells during the period of reduction but remains reduced until reoxidation occurs.

In the lower concentrations of dye in which staining occurs slowly, reduction and reoxidation can be repeated several times on the same material within an hour or two, with the same results in each reduction; but if reduction and reoxidation occur in the dye solution, the most susceptible regions are finally injured by the dye and their reducing power is decreased or lost. If the material is returned to water for reduction after staining, continued entrance of the dye is, of course, avoided.

Reduction in isolated parts, such as tentacles, gonophores, pieces of stem, etc., can be hastened by sealing a number of similar parts in the same cell or by adding pieces or hydranths merely as accelerators of reduction; but in all cases it is necessary to provide for practically continuous change in position of the pieces. Reduction in the various parts of the body has been followed with single isolated pieces and with several pieces in the same cell.

In the figures which show stages of reduction, the depth of color is indicated by depth of shading and the direction in which reduction progresses by arrows. The figures were drawn from individual cases. No attempt is made to show a number of successive stages for the same piece, since reduction becomes evident first in a certain region or in certain regions and progresses in a definitely directed gradient.

DIFFERENTIAL REDUCTION IN MATURE ANIMALS

Animals of all lengths above 25 mm. were regarded as mature, since all such individuals have developed gonophores and may be sexually mature.

Intact hydranths.—With intact hydranths of mature animals the close approximation to each other of the proximal parts of the numerous tentacles and the numerous gonophores crowded together made it impossible to obtain uniform oxygen decrease in the fluid about the hydranth, even with continuous movement. If intact hydranths are left undisturbed for a few moments in dye solution, these parts may either reduce the dye as rapidly as it enters or, if already stained, reduce more or less completely; and reoxidation occurs only when the hydranths are again moved about or when the parts which show no color are isolated from each other. Apparently, when the hydranth remains undisturbed for a few minutes there arises about the hydranth body a zone of lower oxygen content than elsewhere in the medium, in consequence of oxygen consumption by the mass of tissue. Near the hydranth body the oxygen content very soon becomes low enough to permit rapid reduction of methylene blue in open preparations. Oxygen content increases with increasing distance from the hydranth body. Under these conditions the hydranth body and the parts nearest to it reduce earlier than more distant parts. For example, the proximal portions of the long proximal tentacles do not stain, or reduce rapidly, while the distal portions which extend far from the body do not reduce at all except in sealed preparations. In intact animals in standing water, almost continuous movements of the closely crowded distal tentacles, rhythmical contractions of the gonophore umbrellas, extension and contraction of the gonophore stalk and manubrium go on almost continuously; and, somewhat less frequently, changes in position of the proximal tentacles, contraction, extension and bending of the stem occur. It

appears probable that at least some, if not all, of these movements are induced by decrease of oxygen about the hydranth body; and there can be no doubt that they result in a more uniform distribution of oxygen to the crowded parts. The hydranths are highly sensitive to lack of oxygen. When sealed in a small volume of sea water, they lose motility in a short time (and death follows if oxygen is not readmitted), but recovery of motility may occur on exposure to air. The very slight change in pH under these conditions indicates that lack of oxygen, not accumulation of CO_2, is the effective factor. If hydranths are cut off and allowed to lie on the bottom of the container in standing water at room temperature, they begin to die and disintegrate in a day or less; but if kept in circulation in the container by a gentle current, they remain in good condition for days. Small young animals which have not yet developed large numbers of tentacles and gonophores, when kept in standing water with occasional change, apparently die only from starvation or lack of proper food. Because of the difficulty of maintaining uniform oxygen distribution and because of differences in rate of staining, reduction gradients observed in intact hydranths of mature animals are in part of uncertain significance but are briefly described in order to call attention to some of the complicating factors that arise, even in forms as simple as this, and for comparison with results obtained on isolated parts of the hydranth and early developmental stages.

In all concentrations of dye from 1/200,000 to 1/20,000, distal and proximal tentacles stain more rapidly and are also more susceptible to toxic effect of the dye than any other parts. Both staining gradient and susceptibility gradient are basipetal in the distal half or two-thirds of the tentacle; but in the lower concentrations (e.g., 1/100,000) the proximal parts of the tentacle do not stain at all, or very slightly. If reduction begins before injury occurs, it progresses in the tentacle ectoderm from the tips basipetally in the distal half or two-thirds of the tentacle, but in the proximal part of the tentacle reduction progresses acropetally. If the hydranths are allowed to lie without change of position after sealing, reduction progresses entirely acropetally in the tentacles. With the beginning of injury at the tentacle tips, reduction is retarded or does not occur there but still occurs farther proximally.

The hypostome stains more rapidly and more deeply than other

parts of the hydranth body. As will appear below, this characteristic is evidently associated with the differentiation of this region and is more or less specific. With slight staining, the hypostome will reduce before more proximal parts of the body; but, when deeply stained, reduction is greatly retarded. Other parts of the hydranth body may remain entirely unstained in 1/100,000 or lower, even in open preparations; and when stained in higher concentrations, show rapid reduction often in open preparations. The medusoid gonophores are so crowded that, except where fully exposed to the medium, they either do not stain at all or, if stained, reduce rapidly when sealed—most rapidly in the most crowded parts. When the gonophore is uniformly exposed and slightly stained, reduction begins at the tip of the manubrium and progresses proximally in the ectoderm. With deeper staining, injury and retardation of reduction occur, first at the tip, and progress proximally. The gonophore umbrella stains more rapidly than the manubrium and is highly susceptible and readily killed by the dye, but, if not injured, seems to show slightly more rapid reduction at the margins.

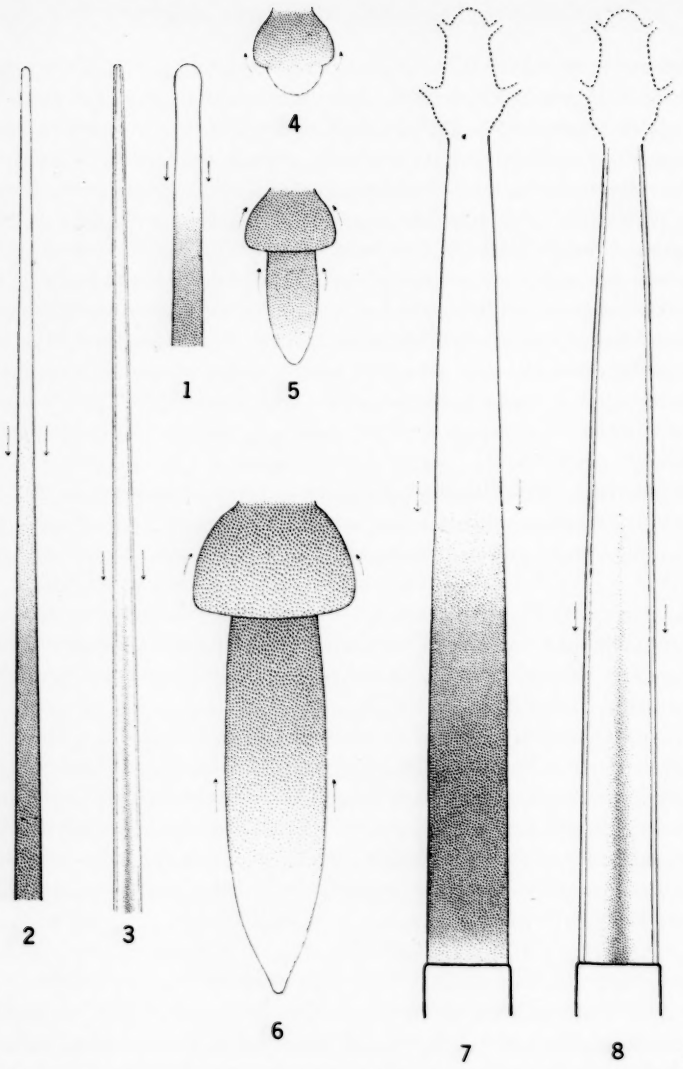
Isolated tentacles.—Distal tentacles removed from the manubrium and stained 2–3 minutes in 1/25,000 methylene blue show apparently uniform staining of ectoderm from tip to proximal cut end, with little or no staining of entoderm. If the solution is then diluted to 1/100,000 or 1/120,000 to prevent too rapid further staining, or if the tentacles are returned to water and a number sealed together, reduction appears first at the tentacle tips and progresses basipetally in the stained ectoderm (Fig. 1). As oxygen in the preparation decreases, reduction and reoxidation of the dye can usually be observed repeatedly without exposure to air. When the tentacles are not moved about in the medium for a few minutes, basipetal reduction occurs. If the tentacles are then moved about in the fluid by changes in position of the slide with respect to gravity, reoxidation occurs; and basipetal reduction again takes place when they again remain undisturbed for a short time. Evidently, when not moved about in the solution, the decrease in oxygen immediately about the tentacle soon reaches the point at which reduction of the dye in the ectoderm begins; and reduction proceeds more rapidly at the tip of the tentacle and progressively slower at more proximal levels. When the tenta-

cles are moved into other regions of the solution where the oxygen content is somewhat higher, reoxidation takes place, followed by basipetal reduction as each tentacle again decreases the oxygen in its immediate environment. Finally the oxygen content in all parts of the solution becomes so low that no reoxidation occurs with change in position of tentacles. On exposure to air, however, the color reappears rapidly; and it can usually be observed that reoxidation progresses from the proximal region of the tentacle acropetally. This acropetal color gradient which is visible for only a few seconds, suggests that oxygen concentration in the cells attains the level at which reoxidation of the dye can occur earlier in the proximal than in the distal regions. Since reduction of the dye occurs earlier in the distal than in the proximal region of the tentacle, the two lines of evidence support each other.

The results with the much longer proximal tentacles are essentially similar. Isolated tentacles stained 2–5 minutes in 1/25,000 methylene blue show either uniform staining of ectoderm or a slight decrease in depth of color basipetally, and no stain in entoderm or slight staining at and near the tip. In sealed preparations in 1/100,000 methylene blue or in water, reduction progresses basipetally in the ectoderm (Fig. 2) and reoxidation usually shows temporarily a slight acropetal gradient.

On reoxidation after reduction the tentacle entoderm, which is a solid core of cells, is usually also stained. If reduction is repeated, entodermal reduction occurs later than ectodermal reduction but also progresses basipetally and occurs first next to the ectoderm, the deeper parts of the entoderm at any level being the last to reduce. Consequently decoloration occurs last in the deepest parts of the entoderm of the proximal tentacle region (Fig. 3). The course of reduction from the superficial to the deeper parts of the entodermal core suggests that higher oxygen consumption of ectoderm is a factor in determining entodermal reduction. In fact, it remains uncertain whether the basipetal reduction gradient of the tentacle entoderm is intrinsic or merely a secondary result of the ectodermal gradient.

In tentacles placed in low concentrations of dye (1/200,000–1/500,000) and sealed at once, the gradient of staining is usually



FIGS. 1–8.—Differential reduction in parts of mature animal. Figure 1, ectodermal gradient in distal tentacle; Figure 2, ectodermal gradient in proximal tentacle; Figure 3, optical section of proximal tentacle showing differential reduction in entoderm; Figures 4–6, Differential reduction in gonophores; three developmental stages; Figure 7, ectodermal reduction gradient in intact stem; Figure 8, optical section of stem showing differential reduction in supporting cells.

more or less acropetal in the ectoderm; and the ectoderm at and near the tentacle tips may not become stained at all, even though the entoderm in that region gradually stains. The only possible conclusion seems to be that under the conditions of this experiment distal regions of the tentacle ectoderm reduce the dye as rapidly as it enters, while more proximal levels reduce it less rapidly and become more or less stained. With progressive decrease of oxygen in these preparations the usual basipetal reduction gradient appears in the parts which are stained.

The reduction gradient in tentacle ectoderm can be observed in a wide range of dye concentrations. The chief precaution to be observed is adjustment of staining period preceding reduction to dye concentration. If staining is too long continued, deep staining and injury result, beginning at the tip and progressing basipetally. When such injury occurs, the reducing power of the injured region is decreased or destroyed and the injured region remains deeply stained, while proximal to it the basipetal reduction gradient may still appear. Under the usual conditions of staining, cytolysis and disintegration of the tentacles following injury by the dye also begin at the tip and progress basipetally.

Gonophores.—Isolated gonophores in various stages of development after 3–4 minutes in 1/12,500–1/25,000 methylene blue show the dye in the ectoderm of the manubrium and in the umbrella. In the smaller gonophores in earlier stages of development the whole thickness of the manubrium ectoderm is usually stained but the entoderm remains unstained. In the larger, full-grown or nearly full-grown gonophores, staining is usually limited; the outer more superficial parts of the manubrium ectoderm are stained, but the deeper parts do not usually show visible staining and the entoderm is not stained. If the gonophores are sealed in a lower concentration of dye (e.g., 1/100,000) in order to prevent too rapid further staining, reduction in most individuals occurs first at the tip of the manubrium ectoderm and progresses basipetally (Figs. 4–6). When the whole thickness of ectoderm is stained, reduction at a given level of the manubrium usually appears to occur first in the deeper parts of the ectoderm and to progress toward the external surface. This differential suggests the possibility that either the entoderm or the deeper

parts of the ectoderm may have a higher oxygen consumption than the superficial parts. Even with brief periods of staining, however, a few individuals may show slightly deeper staining and slower reduction at the extreme tip of the manubrium, with basipetal progress of reduction at more proximal levels. With this procedure reoxidation and reduction can usually be repeated two or three times in dye solutions, but with each repetition of reduction a larger number of individuals show a region of slower reduction at the manubrium tip and extension basipetally of this region. With increase in the period of staining preceding reduction this region of slower reduction at the manubrium tip also appears more frequently and extends farther basipetally until the proximal parts of the manubrium reduce the dye most rapidly and reduction is increasingly retarded toward the distal end. In other words, the basipetal reduction gradient which appears with slight staining may be reversed with deeper staining. Finally, with continued staining cytolysis and disintegration begin at the tip of the manubrium and progress basipetally.

The umbrella of the gonophore stains rapidly and is injured and killed by a given concentration of dye earlier than the manubrium. After slight staining, however, reduction seems to occur slightly earlier in the marginal region than elsewhere and to progress from the margin to the region of union with the manubrium.

The hydranth body.—It has not been possible thus far to obtain satisfactory results with the hydranth body. When the tentacles are removed as completely as possible, their cut bases remain on the manubrium and in the proximal region and may affect conditions in the body. After removal of tentacles and with very slight staining, the ectoderm of the hypostome distal to the distal tentacles seems to reduce slightly more rapidly than more proximal regions; but with further staining, the hypostome becomes more deeply stained and reduces more slowly than any other part of the hydranth body. Both the intertentacular region of the manubrium and the post-tentacular region of the hydranth reduce rapidly, the former probably a little more rapidly than the latter. When stained, hydranth entoderm reduces much later than ectoderm.

The naked stem region.—In the mature animal four-fifths or more of the stem is naked, only the basal portion being covered by peri-

sarc. For observation of reduction in the naked region the tangle of holdfasts attached to sand grains was cut away at the base, and either the hydranth was left attached or all except the post-tentacular part of the hydranth body was removed. The ectoderm of the naked stem region stains uniformly over the whole length, or a very slight basipetal staining gradient appears. Reduction becomes evident first in the distal region and progresses basipetally over the naked region almost to the perisarc (Fig. 7). Immediately distal to the perisarc there is in many stems a short, rather sharply localized region in which reduction occurs somewhat more rapidly than in other parts of the proximal region. The short basal region inclosed in perisarc stains very slowly or not at all, apparently because the dye penetrates the perisarc very slowly. Some slight indications of a reduction gradient opposite in direction to the gradient of the naked stem have been observed but are not regarded as conclusive.

It was found that with 5–15 minutes' staining in 1/20,000 methylene blue the stain is deep enough to show very clearly the basipetal reduction gradient in the ectoderm. The stems usually remained in the same concentration during reduction, but in some cases they were stained in 1/20,000 and sealed in a lower concentration. Time of appearance of reduction varies with volume of fluid and size and number of stems in the sealed preparation, but reduction can be made to appear within 5–10 minutes after sealing.

On readmission of oxygen after reduction, the color returns rapidly in an acropetal gradient; that is, it appears first in the more proximal part and progresses distally. This gradient is visible for a short time—a minute of two—and gradually disappears. This acropetal reoxidation gradient suggests that the intracellular oxygen concentration which permits reoxidation is attained earlier in the proximal regions, which reduce more slowly than in the more rapidly reducing distal regions; and this in turn suggests a higher oxygen consumption distally than proximally.

With the procedures described, reduction and reoxidation have been repeated two, three, and four times on the same material, with the same results in each case, and could doubtless be still further repeated.

If the stems are sealed in a dye solution for reduction, the dye

continues to enter during the reduction period, in reduced form; but on reoxidation, the stain is deeper than before reduction. Repeated reduction and reoxidation with any concentration desired make it possible to observe the effect on differential reduction of the progress of staining. It is found that the same basipetal reduction gradient appears until the staining becomes very deep. Then injury and retardation of reduction begin to appear in the distal region and progress proximally.

Reduction in the longitudinal entodermal canals just beneath the ectoderm occurs at a given level about the same time as ectodermal reduction. The oxidized dye reaches the large supporting cells which fill the interior of the stem only very slowly, but in reduced form it apparently reaches them more rapidly. With repeated reduction and reoxidation these cells become stained sooner or later. Reduction occurs in them later than in the ectoderm but follows the same basipetal course as in the ectoderm and at a given level progresses from periphery toward the center, that is, the region nearest the more rapidly reducing ectoderm reduces earlier than deeper regions. Consequently, reduction occurs last in the supporting cells farthest from the surface in the proximal region (Fig. 8). It seems probable that the reduction gradient from distal to proximal and from periphery to center in this solid core of supporting cells is not intrinsic, but determined by the gradient in the ectoderm. If there is a basipetal oxygen consumption gradient in the ectoderm and a higher oxygen consumption in the ectoderm than in the supporting cells, the decrease in oxygen in the supporting cells to the level which permits reduction may be determined in part by the oxygen consumption of the adjoining ectoderm, and reduction will occur earlier in the supporting cells of the distal than in those of the proximal region and at any level of the stem earlier in the supporting cells nearest the ectoderm. This is the observed course of reduction in the supporting cells.

The holdfasts.—The holdfasts of *Corymorpha* are very slender stolons which arise from the basal region of the stem. They grow out into the mud, secreting perisarc as they grow; and after they have attained a certain length, the tip continues to grow at the expense of more proximal levels, which evidently serve as nutrition for the

growing tip and gradually disappear so that cellular continuity with the stem is lost and only perisarcal continuity remains. Growth of the stolon tips at the expense of mere proximal levels continues until they are reduced to very small size and available material for growth is exhausted (Child, 1928).

In order to obtain holdfasts free from mud which adheres to the perisarc in the normal habitat, stems with extreme basal ends and holdfasts removed were placed in containers with slides on the bottom. New holdfasts develop and grow to a considerable length, often 1–2 cm. on the slides within 24 hours. Since the perisarc adheres to the glass, staining and reduction can be carried out without removal from the slide and while growth is going on. The extremely delicate perisarc apparently delays staining somewhat but does not prevent it. After staining for several hours in 1/100,000 and 1/50,000 and after 15 minutes in 1/20,000 methylene blue, the terminal portions of the holdfasts which contain living cells and extend several millimeters from the tip are stained throughout. In addition to a general diffuse staining, numerous deeply stained granules or masses of various size appear chiefly or wholly in the entoderm. In the extremely slender parts farther from the tip which apparently contain cellular débris, only scattered granules stain. After sealing, reduction occurs first at the tip and progresses basipetally and from ectoderm to entoderm. The deeply stained granules in the entoderm reduce less rapidly than other parts of the cells. Figure 9 shows three stages of reduction. In the slender parts farther from the tip the scattered granules reduce very slowly or not at all, and no definite gradient appears.

DIFFERENTIAL REDUCTION DURING RECONSTITUTION

Differential reduction of methylene blue has been followed in isolated stem pieces during reconstitution by both of us, largely independently, with complete agreement in results obtained. A wide range of concentration of methylene blue was used in preliminary experiments; but 1/20,000 with a staining period of 10–15 minutes preceding sealing was used in comparative experiments because, with this concentration and staining period, staining and reduction required only 30–45 minutes and reoxidation and reduction could be

repeated several times with the same pieces without any evident change in condition or toxic effect. Pieces 15–20 mm. in length from the distal stem regions of 40–50 mm. animals were used as standard pieces. Such pieces almost invariably develop as bipolar forms, that is, with a hydranth at both cut ends, the distal hydranth usually developing slightly more rapidly than the proximal (Watanabe,

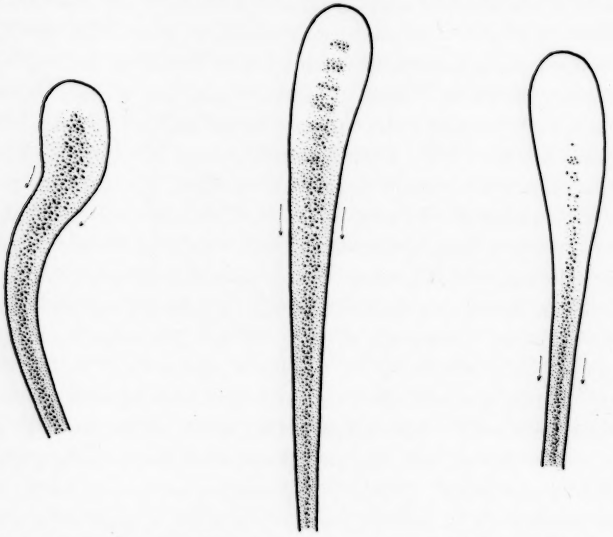
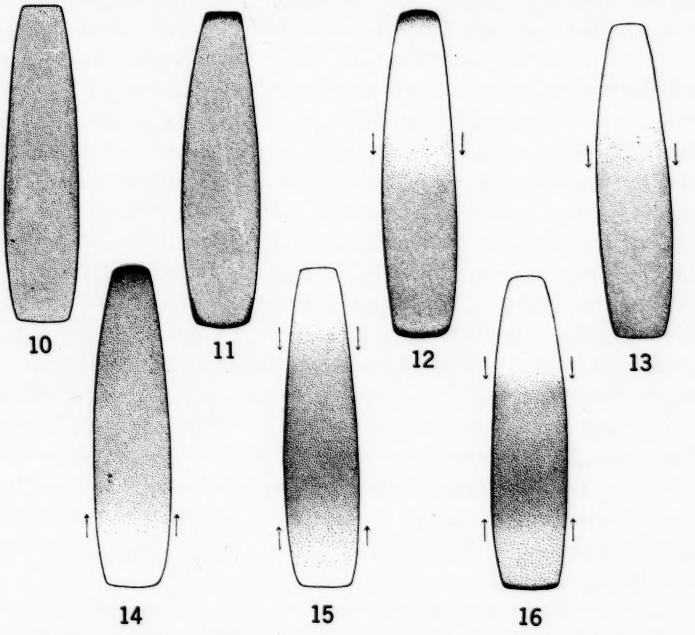


FIG. 9.—Three stages of differential reduction in terminal parts of holdfasts

1935, Figs. 1–8). In most cases the distal hydranth is determined on a slightly larger scale of organization than the proximal, but changes in diameter and length of the two hydranth regions in consequence of extension and contraction often make it difficult to determine whether difference in scale of organization exists.

After closure of the cut ends staining of ectoderm is, so far as can be determined, practically uniform over the whole length of the piece. The longitudinal entodermal canals which lie immediately beneath the ectoderm are usually also more or less stained; but the entodermal parenchyma stains only with considerably longer staining periods or after reduction and reoxidation in the dye solution,

which, of course, amounts to essentially the same thing as a longer staining period preceding reduction. Differential reduction in these pieces is clearly visible to the naked eye, but the pieces were also examined microscopically by transmitted and reflected light. Staining and reduction were observed in numerous single pieces at various periods up to 48 hours after section, but for purposes of record four lots of 20 pieces each were observed.



FIGS. 10–16.—Variations in differential reduction in standard pieces following section with 12–15 minutes' staining in 1/20,000 methylene blue and reduction in 15–30 minutes.

In the first lot with staining begun immediately after section, and reduction completed 30–45 minutes later, considerable variation in the course of reduction appears (Figs. 10–16). In 14 of the 20 pieces a region of more rapid and deeper staining and slow or no visible reduction appeared at either distal or proximal cut end (Figs. 11, 12,

14, 16). This region is sharply localized close to the cut end. It was found, however, that with lower concentrations and short periods of staining, these regions did not stain more deeply than others and showed rapid reduction. When not overstained, they reduce more rapidly than other parts; but with longer staining or higher concentration, they are injured earlier than other parts, become deeply stained, and reduce very slowly or not at all. They evidently represent early activating effects of section on the cells near the cut ends. The course of reduction also differs in other parts of the pieces immediately after section. Of the 20 pieces, 4 showed no differential (Fig. 10), 4 showed the basipetal reduction gradient like intact stems (Figs. 12, 13), 6 showed a reversed (acropetal) reduction gradient (Fig. 14), and 6 a double or bipolar reduction gradient, that is, reduction progressed from both ends (Figs. 15, 16).

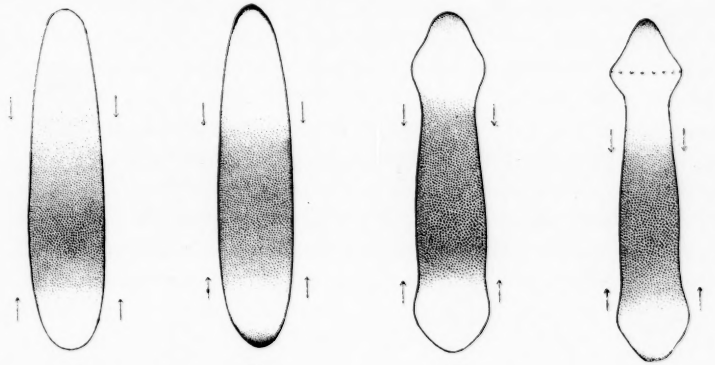
These variations in staining and reduction following section apparently result from individual differences in physiological condition in different pieces. Following section, changes are going on in the pieces which result in determination of certain regions as hydranth regions and later in reorganization and development of hydranths. These changes undoubtedly occur more rapidly in some pieces than in others. With the concentration and staining period used, staining and reduction require a half hour or more following section; consequently somewhat different stages of the changes following section may be expected to appear in different pieces.

Since the deeply stained regions which appear at one or both ends of some pieces (Figs. 10, 12, 14, 16) are, with lower concentrations, the regions of most rapid reduction but are overstained and injured, in 1/20,000 it is evident that Figures 11, 12, 14, 15, and 16 all show some stage of bipolar reduction. In Figures 15 and 16 the bipolar reduction gradients characteristic of later stages are already well developed; Figures 11 and 12 show earlier stages in the determination of these gradients. In Figure 13 the proximal gradient had not yet appeared, while in Figure 14 it is slightly in advance of the distal gradient.

In the lot stained 5 hours after section the terminal regions of slow reduction did not appear in any case in the first reduction (Fig. 17), but in some pieces they appeared at one or both ends when reduction

was repeated three or four times in dye solution, with, of course, deeper staining preceding each successive reduction (Fig. 18). This result again shows that these terminal regions are primarily regions of rapid reduction but are more susceptible to toxic action of the dye than other parts.

In all 60 pieces of the three lots stained 5, 24, and 30 hours after section, a double or bipolar reduction gradient appeared distinctly (Figs. 17 and 18, 5 hours; Figs. 19 and 20, 24 hours). As soon as the

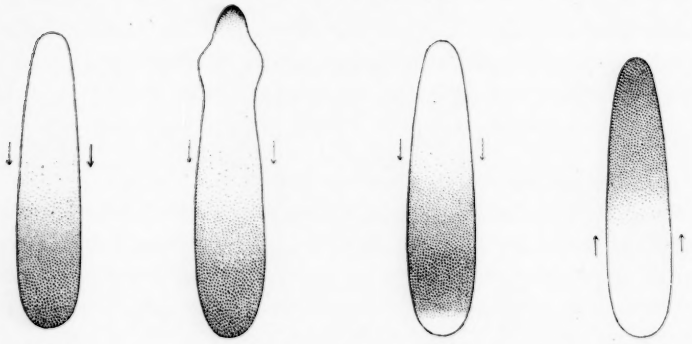


FIGS. 17–20.—Bipolar reduction gradients in standard pieces. Figure 17, first reduction 5 hours after section; Figure 18, repeated reduction and reoxidation in dye solution 5 hours after section; Figures 19 and 20, reduction 24 hours after section.

hypostome region reaches a certain stage of development, it begins to stain more rapidly in the concentration used and reduces more slowly than other parts of the hydranth body (Figs. 19, 20). The terminal regions which become deeply stained and reduce slowly in some pieces immediately after section (Figs. 11, 12, 14, 16) give rise later to the hypostomes; but between the two stages there is apparently a period of lower susceptibility of this region to the dye, in which the deep staining and slow reduction occur only with higher concentrations or longer staining periods. With very low concentrations and slight staining, the hypostome reduces as rapidly as, or more rapidly than, other regions of the hydranth body.

The early stages of tentacle buds stain rapidly, that is, are ap-

parently highly permeable to the dye and reduce very slowly in 1/20,000 (Figs. 20, 21). They are very highly susceptible to the dye and, even when very slightly stained in very low concentrations, returned to water, and kept in darkness, are strongly inhibited in development, and may even undergo regression to complete disappearance. These characteristics of tentacles and hypostome appear to be in some degree specific for these organs, and certainly appear only when a certain developmental stage is attained.



FIGS. 21–24.—Unipolar reduction gradients. Figures 21 and 22, completely unipolar reduction with hydranth region at distal end; Figure 23, unipolar reduction with distal hydranth region and limited proximal reduction (inhibited proximal gradient?); Figure 24, unipolar reduction with hydranth region proximal and complete reversal in direction of reduction gradient.

Unipolar development occurs only rarely in these pieces in normal environment; but under certain experimental conditions, to be described in the following paper (Watanabe, 1935), frequency of unipolar development is high, and the position of the hydranth can be determined either at the original distal or proximal end of the piece. Other experimental conditions also make possible determination of the hydranth at either end of the piece or even elsewhere (Child, 1926*b*; 1927*a*, *b*, *c*).

The reduction gradient in unipolar forms 5 hours after section is shown in Figures 21–24. In Figures 21–23 the hydranth develops at the distal end. Figure 23, however, shows a short, rather sharply

limited region of early reduction at the extreme proximal end. This appears in some unipolar pieces and probably represents an inhibited gradient which does not attain conditions resulting in hydranth development, but is perhaps concerned later in development of a basal end. In Figure 24 the hydranth will develop at the original proximal end, and the gradient is reversed throughout the whole length of the piece (see also Watanabe, 1935, Figs. 31, 32).

When reoxidation occurs following reduction in the pieces, whether bipolar or unipolar, temporary gradients of return of color opposite in direction to the reduction gradients are almost always visible for a few seconds; and if the fluid is not stirred or agitated to bring about rapid oxygen increase, such reoxidation gradients may persist a minute or two or even longer. If the pieces are not moved about during staining when open to air, the hydranth region at 8–16 hours usually shows less color than other regions; but when the pieces are moved about, color immediately increases. In the sealed preparation reduction occurs very early in the hydranth region—during this period of development much earlier than in other parts. It is often visible within 5 minutes after sealing, even when 6–8 pieces are sealed in 2 cc. of solution. When pieces are sealed immediately after being placed in dye solution, the hydranth regions often do not stain at all but reduce the dye as rapidly as it enters while other parts stain. On opening such preparations, the hydranth regions rapidly become more or less stained.

When pieces undergo a second or third reduction under the same conditions as the first, these reductions usually occur in less time than the first, provided the staining has not yet reached the point at which definite toxic effects appear. In some cases after reoxidation the hydranth region remains almost unstained or only slightly stained in the open preparation, while other parts of the piece show deeper color. It is a well-known fact that oxygen consumption is increased in various organisms by methylene blue, and the observations noted above suggest an increase in *Corymorpha;* but evidence must await determinations of respiration.

These data on differential reduction show that immediately following section the basipetal reduction gradient of the intact stem may be present or may be obliterated or partially or wholly reversed.

Evidently the gradient is disturbed and altered by section, and during the period of staining and reduction is gradually changing to the definitive form. Within a few hours after section, the gradient or gradients in the piece are definitely related to the region or regions where hydranths will develop. In connection with this condition, immediately following section it may be noted that considerable variations in CO_2 production have been observed following section in determinations of CO_2 production in *Allobophora foetida* (Watanabe, 1931) and the polyclad *Stylochus ijimai* (Watanabe and Child, 1933).

When the dye first enters the tissues, the cells appear to be uniformly and diffusely stained but evidently accumulate the dye to concentrations much higher than in the external solution; but even if the animals or pieces are returned to water after staining, a gradual change in distribution apparently occurs. The dye becomes more or less localized in deeply stained granules or masses, which often appear opaque black by transmitted light. These granules appear to be more numerous and larger in the entoderm than in the ectoderm. After they become deeply stained, they reduce more slowly than other parts of the cells; and if staining is continued, the cells with deeply stained granules appear to be injured and reduce less rapidly than in earlier stages of staining. In general the deeply stained granules become visible first in the regions which, with less staining, reduce the dye most rapidly; and these regions show apparent injury and retarded reduction earlier than other parts. In other words, the gradient in time of appearance of these granules seems to be in the same direction as the reduction gradient.

CONCLUSION

Extended discussion of the data is unnecessary. The most important points are that within 5–8 hours after section the region where a hydranth will develop and the stem for a certain distance proximal to it appear as a reduction gradient with rate of reduction above that of the intact stem at the same level. Evidently even in this short period the changes leading to hydranth development have begun and appear as a reduction gradient with high end where the apical region of the hydranth will develop. In bipolar forms the reduction gradient appears at both ends of the piece; but the proximal

gradient, which represents a reversal of the normal stem gradient, usually appears a little later and is a little shorter than the distal gradient, which is in the same direction as the original stem gradient; consequently, the last region of the stem to reduce is somewhat proximal to the middle. In unipolar forms, only a single gradient is present except at the extreme end, which is determined as proximal where a short region of more rapid reduction, which seems to be an inhibited gradient, appears in some pieces. Whether the hydranth develops at the distal or the proximal end of the piece in the unipolar form, the high end of the reduction gradient is the hydranth region.

Before the form of the hydranth is visible, the hydranth region is distinguishable from the rest of the piece as a region of more rapid reduction. The hypostome region, when it attains a certain stage of development, and the tentacles, when they appear, become regions of rapid staining and high susceptibility to the dye. In the mature animal the hydranth region remains a region of more rapid reduction than the stem, and the stem gradient is in the direction determined in the early stages.

All parts of the body reduce methylene blue except when injured by overstaining, but in certain parts reduction becomes evident earlier and proceeds more rapidly than in others. Apparently differences in oxygen consumption determine differences in oxygen demand as oxygen content of the medium decreases, and these differences must be a factor in determining the differentials in methylene blue reduction.

SUMMARY

1. The intact mature hydranth of *Corymorpha palma* presents certain difficulties to the determination of methylene blue reduction differentials. Isolated distal and proximal tentacles show a basipetal reduction gradient in the ectoderm. Tentacle entoderm reduces more slowly but also shows a basipetal and an axipetal reduction gradient which is probably determined by conditions in the ectoderm. Manubria of the gonophores show a basipetal reduction gradient. The gonophore umbrella gradient is apparently from margin to center. The reduction gradient in manubrium and hydranth body is probably basipetal but is perhaps altered by motor activity.

2. The reduction gradient in the intact naked stem is basipetal

except for a region of more rapid reduction immediately above the perisarc in many stems.

3. Reduction in pieces as soon as possible after section shows disturbance of the original gradient, but within 5–8 hours after section (22° C.) the reduction gradients or gradient present are definite with hydranth region at the high end. In bipolar forms the gradient related to the proximal hydranth region is opposite in direction to the original stem gradient. It usually develops slightly later than the distal gradient and extends over somewhat less than half the length of the piece. In unipolar forms, whether the hydranth is distal or proximal, a single reduction gradient extends over the whole length of the piece, except for a short region of more rapid reduction which appears at the end determined as proximal in some pieces and is probably an inhibited gradient.

4. As hypostome and tentacles develop, they become regions of more rapid staining than other parts of the hydranth.

5. In a region which is injured by the dye, reduction is retarded or does not occur. Injury appears to be associated, at least in time, with a localization of the dye in or on certain cell constituents.

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PHYSIOLOGICAL DOMINANCE IN CORYMORPHA PALMA IN RELATION TO RECONSTITUTION AND METHYLENE BLUE REDUCTION[1]

(Thirty-two figures)

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EARLIER experiments on the development of small grafts implanted laterally in the stem of *Corymorpha palma* have shown that the hydranth exercises a dominance or control of some sort over regions basal to it and through this dominance is able to prevent induction of a new axis by the graft (Child, 1932). The present paper is a further study of dominance in *Corymorpha* and deals first with the dominance of the fully developed and developing hydranth, as shown in certain experiments on reconstitution of pieces, and second, with the differential reduction of methylene blue by the pieces in relation to dominance and to the gradients determined by dominant regions.

The data presented below were obtained during the summer of 1934 at the Scripps Institution of Oceanography, La Jolla, California. To Dr. T. Wayland Vaughan, director of the Institution, and to other members of the staff, acknowledgment is due for the laboratory facilities provided and for many personal kindnesses. The writer wishes also to express his sincere thanks and appreciation to Professor C. M. Child, who has given many helpful suggestions and criticisms during the course of the present work.

MATERIAL AND METHODS

Material for the experiments consisted of pieces 15–20 mm. in length from the distal stem regions of *Corymorpha* individuals 40–50 mm. in length. Pieces of these lengths were chosen because they almost always develop hydranths at both ends under normal condi-

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tions and because this development can be experimentally controlled and altered in definite ways.

Experiments on reconstitution.—Control of the polarity of the piece by the original hydranth and by the developing hydranth at different stages of reconstitution was examined by delaying either the distal or proximal section for different lengths of time after the first section and by making a second distal or proximal section or both at different periods. The various operations are described in connection with particular experiments.

For the purpose of the experiments it was necessary to distinguish the original distal and proximal ends. Local staining was avoided because of the possibility that it might alter physiological conditions, and it was found to be possible to distinguish distal and proximal ends in other ways. The stem increases in diameter from distal to proximal end of the naked region, which constitutes four-fifths or more of its length in the animal used, and this increase is greatest in the distal region which was used for experiment. In the earlier stages of reconstitution the original distal and proximal ends can usually be distinguished by the difference in diameter, but contraction and change in diameter during reconstitution often make the distinction impossible in later stages. Another characteristic difference between the two ends of the pieces used for experiment is that in normal environment the hydranth at the distal end develops a little more rapidly than that at the proximal end. This difference is most clearly marked in the earlier stages of reconstitution and decreases as development progresses, but is still evident 40 hours after section, when hydranth development is far advanced. When either distal or proximal section is delayed, the absence of a hydranth at one end or the difference in stage of development of the two hydranths make the identification of original distal and proximal ends easy. In the experiments in which identification of the two ends might be difficult, only five pieces were placed in each container, and these were examined twice a day or oftener. Bipolar forms were separated as their character became evident. With these frequent examinations certain identification of the original distal and proximal ends was possible. The bowls containing the pieces were submerged in larger containers in running water, and the pieces were moved about several times daily by means of water currents from a pipette.

Results of the experiments on reconstitution are graphed in percentages of unipolar forms as ordinates, plotted against periods of delay of distal or proximal section in hours as abscissae (Figs. 16, 22, 26, 28, and 30).

Differential reduction of methylene blue.—The method used in these experiments was essentially that of Child and Watanabe (1935). A Coleman and Bell preparation of methylene blue "for bacilli" was used. In the experiments described below, methylene blue reduction was used primarily to show changes in condition of the pieces in the earlier stages of reconstitution; and a concentration was chosen from preliminary experiments which stained the ectoderm and the entoderm of the longitudinal entodermal canals of the pieces sufficiently in a short time so that the occurrence and course of reduction could be followed easily in these tissues, but did not stain the entodermal parenchyma which fills the interior of the stem. The concentration used was 1/20,000, and the staining period 12–15 minutes. With this procedure hypostome and tentacles stain rapidly and very deeply even in early stages of their differentiation, and are injured by the dye so that reduction in them is greatly retarded or does not occur at all. This condition is indicated by deep shading of hypostome region and tentacle buds in the figures showing methylene blue reduction in the developmental stages in which it appears (e.g., Figs. 10, 12, 24, 31, 32).

After staining, several pieces were placed in a large tissue-culture depression slide, holding about 2 cc., in the dye solution, under a cover glass with air bubbles excluded, and the preparation was sealed with vaseline. Two or three hydranths were sometimes added to decrease oxygen rapidly. With more or less continuous change in position of the pieces by means of gravity and the consequent stirring of the fluid, local differences in reduction resulting from more rapid oxygen decrease in some regions of the fluid than in others are prevented. The differential reduction of the dye in the pieces is clearly visible to the naked eye, but in all cases the pieces were also examined microscopically both by transmitted and by reflected light. With the concentration and period of staining used, the most uniform results were obtained when reduction was completed or almost completed in 15–30 minutes after sealing.

After complete, or almost complete, reduction in the pieces, the

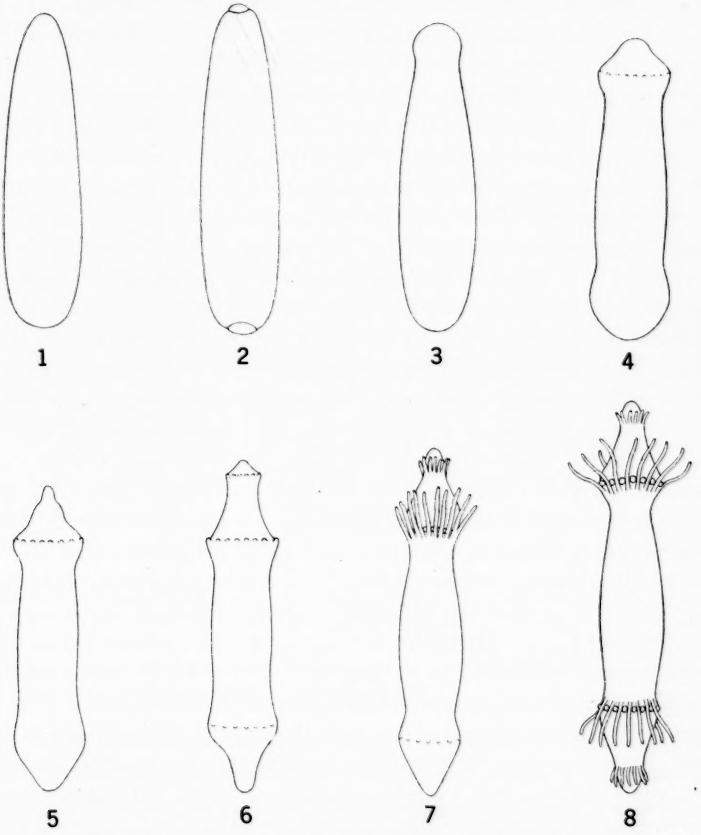
color reappeared in a few seconds on opening the preparation. Usually the pieces were not irreversibly injured by lack of oxygen during the period of sealing and, after a few minutes for recovery, could be sealed and the reduction repeated; but if the pieces are sealed in the dye solution, injury from the dye and retardation or absence of reduction occurs sooner or later. If the pieces are sealed in a much lower concentration of dye, e.g., 1/100,000 or less, or in water, reduction and oxidation of the dye in the tissues may be repeated several times; but, when repeated reduction occurs in water, there is apparently a gradual loss of the dye from the tissues which suggests either that the reduced dye is not as strongly held as the oxidized form or that the organism not only reduces the dye but gradually decomposes it. In order to avoid the temporary changes in condition following section (Child and Watanabe, 1935), staining for reduction was begun 5 hours or more after section.

Figures representing methylene blue reduction are diagrammatic, but from observed cases. Reduction differentials are indicated by depth of shading, the most deeply shaded parts representing regions of least rapid reduction and the unshaded parts, regions in which no color is visible. Arrows represent the direction or directions in which reduction progresses.

RECONSTITUTION OF PIECES AND REDUCTION GRADIENTS

Reconstitution in control pieces.—*Corymorpha palma* has been so extensively used by Child as material for experiments on reconstitution under various conditions (Child, 1926*a*, *b*; 1927*a*, *b*, *c*; 1928*a*, *b*; 1929*a*, *b*; 1932) that only those aspects of reconstitution which are significant for further experiment need be noted here. Pieces 15–20 mm. in length from the distal stem region of animals 40–50 mm. in length, isolated by making distal and proximal section at the same time, constitute the controls for the experiments on delayed section. Such pieces almost invariably develop hydranths at both distal and proximal ends, provided both ends are more or less equally exposed; and almost invariably the hydranth at the proximal end develops slightly later than the distal hydranth. Figures 1–8 indicate the range of variation at various stages up to 48 hours. At 5–8 hours after section the cut ends may be completely closed (Fig. 1) or still

more or less open with entodermal parenchyma protruding (Fig. 2). At 16 hours the distal end usually begins to show some enlargement



FIGS. 1–8.—Reconstitution in standard pieces with distal and proximal section made at the same time. Figures 1 and 2, 8 hours; Figures 3 and 4, 16 hours; Figures 5 and 6, 24 hours; Figures 7 and 8, 48 hours.

(Fig. 3); occasionally proximal tentacle buds are visible on the distal hydranth and the proximal end is beginning to show the form of the hydranths (Fig. 4). Figures 5 and 6 indicate conditions 24 hours, and Figures 7 and 8, 48 hours, after section. The rate of develop-

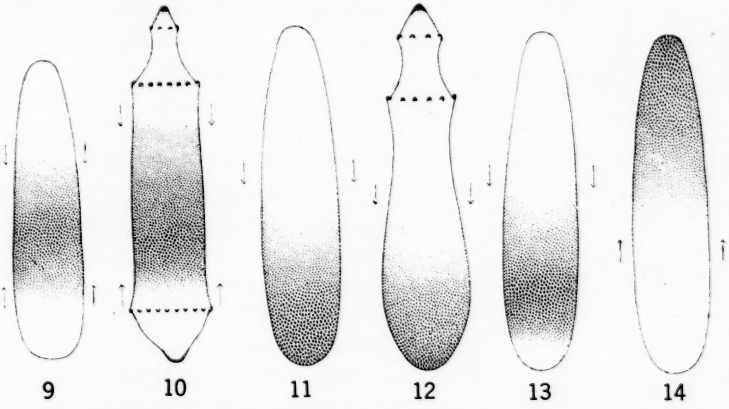
ment indicated in these figures is that observed at the temperature of running sea water in the laboratory which was almost constant at 22° C. during the experiments.

Differential reduction in the intact stem and in control pieces.—The course of reduction in the intact stem and in pieces similar to the controls for the experiments on dominance has been described and figured in the paper preceding this (Child and Watanabe, 1935, Figs. 7, 10–24), so that only the more important points need be noted here. In the intact stem, reduction appears first in the distal region and progresses basipetally over the whole length of the naked region, except for a short localized region of more rapid reduction just distal to the perisarc (Child and Watanabe, 1935, Fig. 7). In the pieces used as controls, this gradient is disturbed and altered immediately after section (Child and Watanabe, 1935, Figs. 10–16); but within 5–8 hours after section and in late stages of development the course of differential reduction is definitely related to regions where hydranths will develop. In pieces which develop as bipolar forms, reduction occurs first at the two ends of the piece and progresses toward the middle. In most cases the distal gradient is a little longer than the proximal, so that the last region to reduce is somewhat proximal to the middle of the piece. Figures 9 and 10 show the reduction gradients as they appear 5–8 hours after section in bipolar control pieces.

Alterations in reduction gradients under experimental conditions.—The control pieces very rarely develop as unipolar forms; but under experimental conditions to be described below, a high frequency of unipolar forms can be determined. Moreover, the position of the hydranth can be determined at either the original distal or the proximal end of the piece. In the unipolar form with hydranth region at the original distal end, a single reduction gradient with earliest reduction in the hydranth region extends over the whole length of the piece (Figs. 11, 12); or, in some cases, a short, rather sharply limited area of more rapid reduction which does not progress distally appears at the extreme proximal end of the piece (Fig. 13). As suggested by Child and Watanabe (1935), this limited region of reduction at the proximal end probably represents the beginning of a gradient which is inhibited in its development by the dominance of the distal region and does not develop beyond this stage, and so does not give rise to

a hydranth, but may perhaps be concerned later in development of a basal end.

In unipolar forms with hydranth region at the original proximal end, reduction also occurs first in the hydranth region and progresses toward the original distal end of the piece. In such cases the original gradient of the intact stem is completely reversed in direction (Figs. 14, 31, 32).



FIGS. 9–14.—Bipolar and unipolar differential reduction of methylene blue in standard pieces. Figures 9 and 10, bipolar reduction; Figures 11 and 12, completely unipolar reduction with hydranth distal, 5 and 24 hours after section; Figure 13, unipolar reduction with hydranth distal, with limited proximal reduction (inhibited proximal gradient?); Figure 14, unipolar reduction with hydranth proximal; Figures 15 and 16, reconstitution with delay in removal of original hydranth.

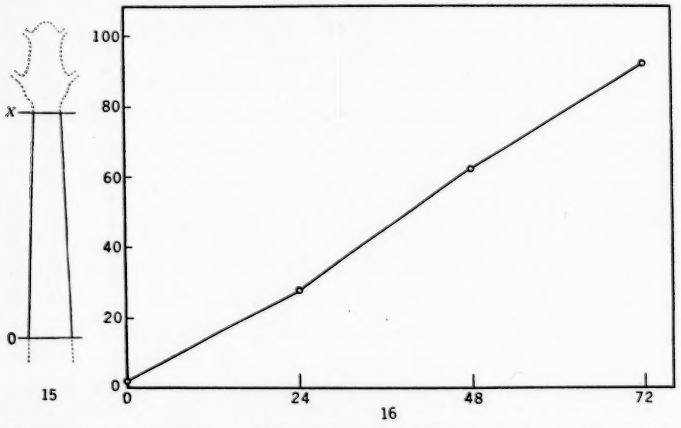
Figures 9–14 indicate the reduction gradients as they appear 5 hours or more after section in the pieces used as standard for control and experiment. They show how closely the development of these gradients is associated with the region of hydranth development.

THE DOMINANCE OF THE ORIGINAL HYDRANTH

Dominance of the original hydranth in reconstitution.—Distal pieces 15–20 mm. long with the original hydranth present and in good condition do not develop hydranths at the proximal end but usually begin to secrete perisarc there a week or more after section, or in some cases the end merely heals and shows no further changes. As noted

above, removal of the original hydranth at the time of proximal section results almost invariably in the development of bipolar forms (Figs. 1–8). These facts indicate that the original hydranth prevents development of a hydranth at the proximal end of the piece.

In order to obtain more definite and more nearly quantitative data concerning this apparent dominance, removal of the original hydranth was delayed 24, 48, and 72 hours after proximal section (Fig. 15), 50 pieces being used for each period of delay. In this ex-



FIGS. 15, 16.—Figure 15, experimental procedure; proximal section at 0 hours, distal section at *X* (0, 24, 48, 72) hours. Figure 16, graph of results; ordinates, percentages of unipolar frequency; abscissae, hours.

periment it is important to keep the hydranths in good condition until their removal. Since the hydranth is heavier than the stem, pieces with the original hydranths kept in standing water always take position with the hydranth downward on the bottom of the container. In this position the hydranths soon begin to degenerate and die, apparently from lack of sufficient oxygen. This degeneration of the hydranths was avoided by keeping the pieces in lots of 10 each in large containers supplied with a continuous water current just sufficient to keep the pieces in circulation and off the bottom.

The pieces with delayed removal of the original hydranth develop either bipolar forms or unipolar forms with hydranth at the distal end, but never a hydranth at the proximal without a hydranth at the

distal end; that is, in no case does a complete reversal of polarity occur. The results are graphed in Figure 16, with percentages of unipolar forms as ordinates and delay of removal of original hydranth in hours as abscissae. It is evident from the graph that the frequency of unipolar forms increases almost uniformly from little above 0 to almost 100 per cent with delay up to 72 hours. The longer the original hydranth remains, the more frequently is development of a hydranth at the proximal end inhibited.

Two additional experiments performed on the dominance of the original hydranth are of interest as supplementing the graphed data. In the first of these, pieces possessing the original hydranths were placed in lots of 10 in standing water with the hydranths in contact with the bottom of the container but with change of water twice daily, instead of being kept away from the bottom by a circulating current. Under these conditions the hydranths soon show evidences of poor condition and sooner or later disintegrate. From 20 of these pieces the original hydranths were removed 48 hours, and from another 20, 72 hours, after proximal section. Of the first 20, with 48 hours' delay of hydranth removal, only 4 (20 per cent) were unipolar; of the second 20, with 72 hours' delay, 9 (45 per cent) were unipolar. Comparison with the graph, Figure 16 shows that these percentages of unipolar forms are much lower than when the hydranths were maintained in good condition until removal. With 48 hours' delay the graph shows 62 per cent; with 72 hours' delay, 92 per cent. Evidently the effectiveness of the original hydranth as a dominant region inhibiting development of a proximal hydranth depends to a considerable degree on its physiological condition.

FIG. 17.—Second proximal section; procedure. First proximal section, 0 hours; distal section and second proximal section, 72 hours.

In the second supplementary experiment the original hydranth in good condition was removed 72 hours after proximal section and at the same time a second proximal section was made 2–3 mm. distal to the first (Fig. 17). Of 20 pieces, 18 (90 per cent) developed hydranths at both ends. This experiment shows that if the proximal

end is activated by a new section, even after 72 hours' subjection to the dominance of the original hydranth it is able to develop a hydranth, provided the original hydranth is removed at the same time.

Dominance of the original hydranth in relation to physiological condition as shown by differential reduction.—In the first of these experiments the pieces were kept in circulation by a current, and the hydranths were in good condition at the time of staining. Lots of 20 or

TABLE I

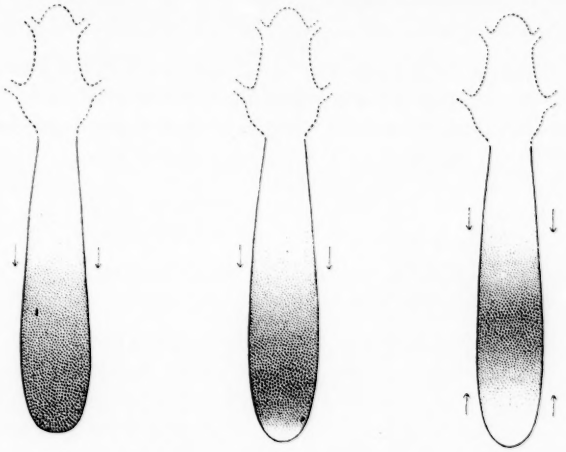
DOMINANCE OF ORIGINAL HYDRANTH AS SHOWN BY DIFFERENTIAL REDUCTION OF METHYLENE BLUE

The first lot (5 hours) consists of 20 pieces; the others, of 10 each.

	Time of Staining after Proximal Section, (in Hours)	Percentage		
		Basipetal	Basipetal with Limited Proximal Reduction	Bipolar
Hydranth in good condition	5	95	5	0
	24	40	60	0
	40	0	100	0
	72	20	80	0
	96	0	100	0
Hydranth in poor condition	5	30	10	60
	48	0	20	80
	72	0	20	80
	88	0	60	40

10 pieces each were stained 5, 24, 40, 72, 96 hours after proximal section. The results of differential reduction are given in the upper section of Table I. The headings of the third, fourth, and fifth columns indicate the course of reduction. "Basipetal" reduction is reduction progressing from distal to proximal end of the piece, as in the intact animal (Fig. 18). "Basipetal with limited proximal reduction" is basipetal reduction over all except the extreme proximal region, where a limited area of more rapid reduction appears (Fig. 19). "Bipolar" reduction is almost equal reduction at both ends with gradients in opposite directions (Fig. 20). As the upper section of Table I shows, the reduction gradient in pieces stained 5 hours

after section is entirely basipetal in 19 (95 per cent); with later staining the limited proximal reduction area appears more frequently but shows no uniform increase with increased delay of staining and reduction. No cases of bipolar reduction appear in this series. These results indicate that, in spite of the presence of the original hydranth, an apparently inhibited gradient may appear at the proximal end, but that this does not lead to hydranth development,



FIGS. 18–20.—Differential reduction with original hydranth present. Figure 18, hydranth in good condition, gradient unipolar and basipetal; Figure 19, hydranth in good condition, gradient unipolar and basipetal except for limited proximal region of more rapid reduction; Figure 20, hydranth in poor condition, bipolar gradients.

though it may perhaps finally lead to development of a basal end. Many pieces of this series begin to secrete perisarc at the proximal end a week or more after section.

The second section of Table I gives results of similar experiments with pieces kept in standing water with hydranths in contact with the bottom and not in good condition. Lots of 10 pieces each were stained 5, 48, 72, and 88 hours after proximal section. The difference of the reduction gradients from those of the first section is striking. Only 5 hours after proximal section and only in 3 pieces (30 per cent) does the complete basipetal gradient appear. Also, the limited proxi-

mal reduction area is less frequent in this series than in the preceding, and bipolar reduction (Fig. 20) shows a high frequency here but does not occur at all in the preceding series. The data of Table I show, first, that the dominance of the original hydranth can be demonstrated in the differential reduction of methylene blue and, second, that the effectiveness of this dominance, as shown by differential reduction, depends to a considerable degree on the physiological condition of the original hydranth.

TABLE II

DIFFERENTIAL REDUCTION OF METHYLENE BLUE WITH DELAYED REMOVAL OF ORIGINAL HYDRANTH

Staining begun 5 hours after hydranth removal. Each lot consists of 20 pieces.

	Delay of Hydranth Removal (in Hours)	Percentage				
		Basipetal	Basipetal with Limited Proximal Reduction	Bipolar	Acropetal	Apolar
Hydranth in good condition	40	5	60	30	5	0
	72	0	80	20	0	0
	96	15	80	5	0	0
Hydranth in poor condition	48	25	15	60	0	0
	88	15	35	25	15	10

Differential reduction with delayed removal of original hydranth in relation to physiological condition.—Two series of reductions with delayed removal of the original hydranth were performed, one with hydranths maintained in good condition by current up to the time of removal, one with hydranths allowed to lie on the bottom of the container in standing water and undergoing degeneration. In the first series the hydranths were removed from 20 pieces, 40, 72, and 96 hours after proximal section; in the second, 48 and 88 hours after proximal section, and the pieces were stained 5 hours after removal of the hydranth. Results are given in Table II. In the first series (first section of Table II) the complete basipetal gradient appears in a few cases, the basipetal gradient with limited proximal reduction

is much more frequent, and bipolar gradients decrease in frequency with increase in delay of removal, as in reconstitution. One case of "acropetal" reduction gradient, that is, reduction progressing distally from the proximal end, indicating proximal hydranth determination, occurs.

In the second series (second section of Table II) the basipetal gradient is somewhat more frequent, the limited proximal reduction much less frequent, bipolar and acropetal gradients more frequent than in the first series, and 10 per cent are apolar, that is, have not developed hydranth gradients, probably because of poor condition. Since the number of pieces is not large, the lower frequency of the limited proximal reduction and the higher frequency of a proximal hydranth reduction region (bipolar and acropetal) as compared with the first series are the chief differences and indicate a less effective dominance in the second series.

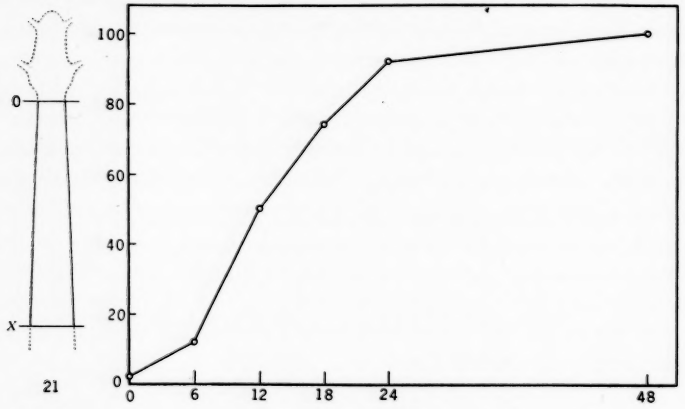
THE DEVELOPMENT OF DOMINANCE OF THE DISTAL HYDRANTH DURING RECONSTITUTION

In the preceding experiments the hydranth concerned is the original fully developed hydranth with dominance already established. Since it may be expected that the hydranth developing on a piece will also become a dominant region, the attempt has been made to obtain experimental evidence concerning the development of its dominance.

Reconstitution and differential reduction with delay of proximal section.—In these experiments the original hydranth was removed and proximal section was delayed 6, 12, 18, 24, and 48 hours, with 50 pieces for each period of delay (Fig. 21). The percentage frequencies of unipolar forms for the periods of delay graphed in Figure 22 show increase from near 0 to 92 per cent with 24 hours' delay and 100 per cent with 48 hours' delay. In these unipolar forms the hydranth is always at the distal end. According to these data, the dominance of the hydranth developing at the distal end of the stem increases so rapidly that with 24 hours' delay of the proximal section hydranth development at the proximal end is almost completely, and with 48 hours' delay is completely inhibited.

For differential reduction with delayed proximal section three lots

of 20 pieces each with delay of 16, 24, and 48 hours, respectively, were stained 5 hours after proximal section. Data of reduction are



FIGS. 21, 22.—Reconstitution with delay of proximal section. Figure 21, experimental procedure; distal section at 0 hours, proximal section at *X* (6, 12, 18, 24, 48) hours. Figure 22, graph of results; ordinates, percentages of unipolar forms; abscissae, hours.

TABLE III

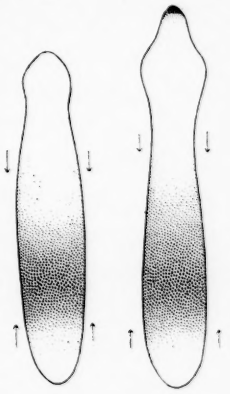
DIFFERENTIAL REDUCTION AFTER DELAY OF PROXIMAL SECTION

Twenty pieces in each lot

Delay of Proximal Section (in Hours)	Percentage			
	Basipetal	Basipetal with Limited Proximal Reduction	Bipolar	Acropetal
16	65	0	30	5
24	95	0	5	0
48	90	10	0	0

given in Table III and agree very closely with the graph. At 16 hours, 65 per cent show a unipolar gradient in the table; in the graph 74 per cent are unipolar in reconstitution with 18 hours' delay. With

24 hours' delay 95 per cent are unipolar in reduction, 92 per cent in reconstitution; and with 48 hours' delay 100 per cent are unipolar in both reduction and reconstitution; but in two cases reduction shows the limited proximal area. Figure 23 shows bipolar gradients with 16 hours; Figure 24, bipolar gradients with 24 hours' delay of proximal section. In these cases the proximal gradient is shorter than in the bipolar form, which develops when distal and proximal section are made at the same time (Fig. 9); and the distal hydranth is, of course, 16 or 24 hours more advanced in development than the proximal.

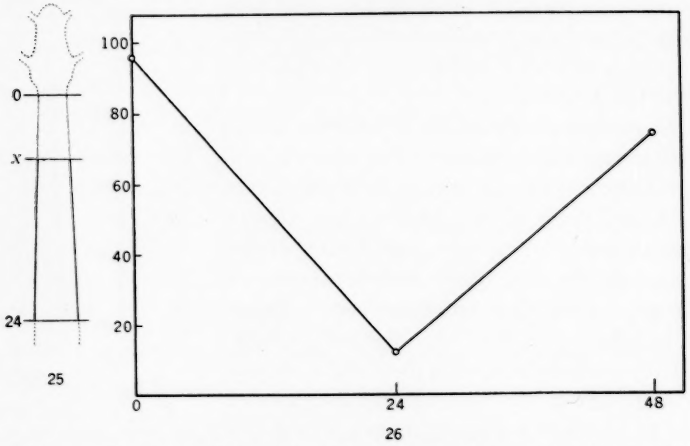


FIGS. 23, 24.—Bipolar reduction gradients after delayed proximal section. Figure 23, 16 hours' delay, and Figure 24, 24 hours' delay, of proximal reduction.

Reconstitution after delay of proximal section with second distal section.—This series is supplementary to the preceding. As shown above, 24 hours' delay of proximal section gives a high unipolar frequency (92 per cent). If this is due to the development of dominance in the new hydranth at the distal end, it should be possible to decrease this high unipolar frequency again by removing the distal hydranth primordium at the time of proximal section and to find it increasing again as a second new hydranth develops.

In order to test this possibility, the original hydranth was removed from 150 pieces, and 24 hours later the proximal section was made. In one lot of 50 pieces the distal region OX, Figure 25, was also removed at the same time as the hydranth. In these the new hydranth developed at X for 24 hours before proximal section was made. In a second lot of 50, section at X was made 24 hours after removal of the hydranth, that is, at the same time as proximal section. In the third lot of 50, section at X was made 48 hours after removal of the hydranth, that is, 24 hours after proximal section. The percentage frequencies of unipolar forms graphed in Figure 26 are high in the first (96 per cent), low in the second (12 per cent), and again high in the third (74 per cent). In other words, when the new hydranth at X (Fig. 25) develops for 24 hours before proximal sec-

tion, development at the proximal end is strongly inhibited; when the distal 24-hour primordium is removed by section at *X* and the proximal section made at the same time, 88 per cent are bipolar, that is, the proximal ends are able to develop hydranths; and when proximal section is 24 hours earlier than section at *X*, the developing hydranth at *O* inhibits development at the proximal end for 24 hours preceding section at *X* and 74 per cent are unipolar.



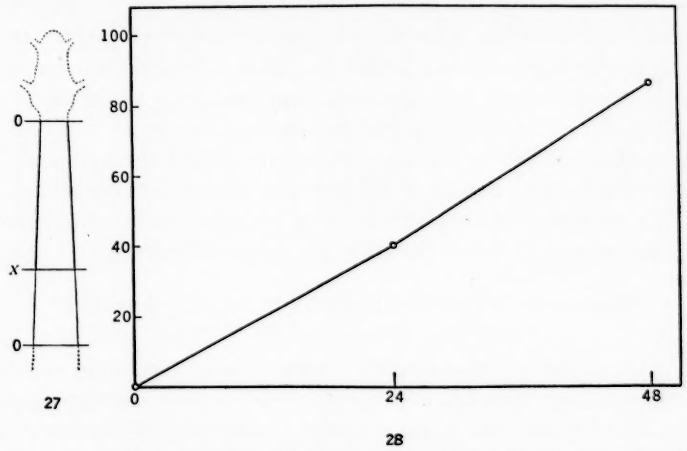
FIGS. 25, 26.—Reconstitution after delayed proximal section and a second distal section at different periods. Figure 25, experimental procedure; distal section, 0 hours; proximal section, 24 hours; second distal section, *X* (0, 24, 48) hours. Figure 26, graph of results; ordinates, percentages of unipolar forms; abscissae, hours.

The first lot of this experiment is similar to the lot of the preceding experiment with 24 hours' delay of proximal section except that in the present experiment the pieces are somewhat shorter. It is shown in the second lot of Table III that the frequency of the unipolar reduction gradient is high in such pieces (95 per cent); consequently, reduction was not observed again in pieces corresponding to this lot.

Reduction in 10 pieces corresponding to the second lot of the reconstitution experiment, that is, with proximal section and second distal section 24 hours after removal of the original hydranth (Fig. 25), showed 10 per cent unipolar, as compared with 12 per cent in reconstitution (Fig. 26), and 90 per cent bipolar reduction gradients.

In 20 pieces like the third lot the reduction differential was determined. Of these, 9 showed complete basipetal gradient, 7 were basipetal with limited proximal reduction; that is, a total of 80 per cent were unipolar, as compared with 74 per cent in Figure 26.

Delayed second proximal section.—This is another supplementary experiment. Distal and proximal section were made at the same time at distal *O* and *X*, Figure 27 in one lot of 50 pieces. In the second and third lots of 50 each distal and proximal section were made



FIGS. 27, 28.—Reconstitution after a second proximal section at different periods. Figure 27, experimental procedure; distal and first proximal section, 0 hours; second proximal section, *X* (0, 24, 48) hours. Figure 28, graph of results; ordinates, percentages of unipolar forms; abscissae, hours.

at the same time at distal *O* and proximal *O*, and a second proximal section at *X* 24 hours later in the one lot and 48 hours later in the other. Percentage frequencies of unipolar forms graphed in Figure 28 show increase from 0 in the first lot to 40 per cent with 24 hours, and 86 per cent with 48 hours' delay of second proximal section. Here, as in preceding experiments, the dominance of the distal hydranth increases as reconstitution proceeds. Comparison of Figure 28 with Figure 22, the graph for delay of a single proximal section, shows that in Figure 28 the increase in unipolar frequency at 24 and 48 hours is much less than in Figure 22. In other words, with 24 and

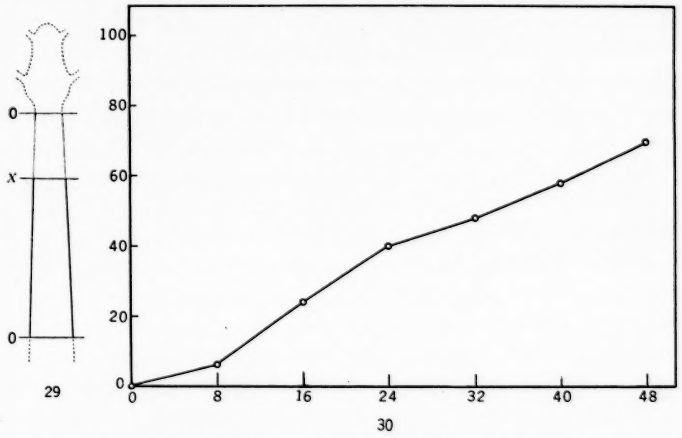
48 hours' delay of the second proximal section at *X* (Fig. 27), many more pieces develop bipolar forms than with 24 and 48 hours' delay of the first proximal section as in Figure 21. Apparently, development of a hydranth primordium at the proximal end of the piece (proximal *O*, Fig. 27) for 24 or 48 hours has altered conditions at *X* to some extent, so that the dominance of the distal hydranth is less effective in inhibiting hydranth development there. Methylene blue reduction shows that in bipolar forms the gradient is reversed from the proximal end almost to the middle of the piece (Figs. 9, 10). The second and third lots of the present experiment developed for 24 or 48 hours as bipolar forms before the second proximal section was made. The evidence from reduction indicates that the gradient at *X* (Fig. 27) was reversed by the development of the primordium at proximal *O*. If this is the case, we should expect the hydranth at distal *O* to be less effective in inhibiting hydranth development at *X* than if reversal of the gradient had not occurred; and comparison of Figures 28 and 22 shows that it is much less effective.

THE DEVELOPMENT OF DOMINANCE OF THE PROXIMAL HYDRANTH DURING RECONSTITUTION

As the preceding experiments show, almost all pieces develop hydranths at both ends when both sections are made at the same time. Consequently, in order to examine the development of dominance of the hydranth at the proximal end, distal and proximal section were made at the same time (distal and proximal *O*, Fig. 29) and a second distal section was made at *X*, one-third of the length from the distal end, at 8, 16, 24, 32, 40, and 48 hours after the first section in lots of 50 for each time. Percentage frequencies of unipolar forms resulting, which are graphed in Figure 30, show a continuous increase in unipolar forms with increase in delay of the second distal section. These unipolar forms, however, have the hydranth at the proximal end. Evidently the dominance of the developing proximal hydranth, as shown by inhibition of development of a hydranth at the distal end, increases as development progresses. Comparison of Figure 30 with Figure 22 shows, however, that this increase is much less rapid than the increase of dominance of the developing distal hydranth, which is 92 per cent at 24 hours and 100 per cent at 48 hours, as com-

pared with 40 and 70 per cent for the same times in the present experiment.

The experiment outlined in Figure 27 and graphed in Figure 28 is, however, more directly comparable with the present experiment. In that experiment distal and proximal section were made at the same time, and a second proximal section removing the proximal primordium was made after different periods of delay. Comparison of the graphs, Figures 28 and 30, shows that the development of dominance

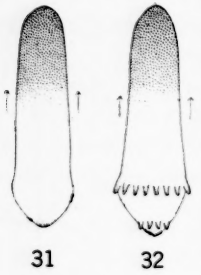


FIGS. 29, 30.—Reconstitution after delayed second distal section Figure 29, experimental procedure; first distal and proximal section, 0 hours; second distal section, *X* (0, 8, 16, 24, 32, 40, 48) hours. Figure 30, graph of results; ordinates, percentages of unipolar forms; abscissae, hours.

of the distal and of the proximal developing hydranth as indicated by the increase in frequency of unipolar forms occurs at almost the same rate. In both cases 40 per cent unipolar forms occur with 24 hours' delay of the second section whether distal or proximal; and with 48 hours' delay of the second proximal section the unipolar percentage is 86 (Fig. 28), while with 48 hours' delay of the second distal section it is 70 (Fig. 30). Apparently, dominance of the new proximal hydranth over the distal end is somewhat less at 48 hours than that of the distal hydranth over the proximal end.

Differential reduction was observed in two lots of 20 pieces each,

with 24 and 48 hours' delay of the second distal section. Of the 24-hour lot, 70 per cent, and of the 48-hour lot, 80 per cent, showed a unipolar reversed reduction gradient, that is, reduction began at the proximal hydranth primordium and progressed to the original distal end (Figs. 31, 32; see also Fig. 14). The others showed bipolar gradients. Evidently the proximal hydranth primordium is increasingly able, as it develops, to reverse the reduction gradient over the whole length of the piece. The 24-hour reduction lot, however, shows 70 per cent of reversed unipolar gradients, while only 40 per cent unipolar appear in reconstitution with 24 hours' delay of the second distal section. This is the only case among all the experiments in which reduction gradients and reconstitution did not show close parallelism. With 48 hours' delay there is closer parallelism—80 per cent reversed reduction gradient, 70 per cent unipolar reconstitution. The reduction and reconstitution experiments were performed with different stocks of material, which may account for the difference.



FIGS. 31, 32.—Reversed reduction gradient after delayed second distal section, proximal hydranth region dominant.

DISCUSSION

Evidence that the original hydranth and the developing hydranth primordium exercise a certain degree of dominance or control of the polarity of the *Corymorpha* stem has been given by Child in studies on reconstitution and induction of new axes by incisions and grafts (Child, 1927*a*, *b*, *c;* 1928*a*, 1929*a*, *b;* 1932). The data of the present paper carry the analysis of dominance in *Corymorpha* somewhat further. They show, first, that the presence of a hydranth at one end of the piece can inhibit hydranth development at the other end and, second, that a new hydranth primordium developing after section gradually becomes dominant as it develops and is increasingly effective up to a certain stage of development in inhibiting hydranth development at the other end of the piece.

In the pieces from the distal region of the naked stem which were used in these experiments, section at both ends at the same time is almost invariably followed by development of a hydranth at both

ends, the hydranth at the proximal end usually developing a little later than that at the distal end.

In the intact animal the ectoderm of the naked region of the stem shows a distinct basipetal (unipolar) gradient in rate of methylene blue reduction in low oxygen. Reduction occurs most rapidly in the distal region of the stem and progresses basipetally. In pieces with distal and proximal section made at the same time, reduction immediately after section shows considerable variation. In some cases no gradient appears; in others, the apicobasal gradient of the intact stem; in still others, a gradient from both ends; and in a few, a gradient from the proximal end (Child and Watanabe, 1935, Figs. 10–16). Considered in the light of the results with later stages, this variation is regarded as indicating temporary disturbance of physiological condition following section (Child and Watanabe, 1935; see also Watanabe, 1931; Watanabe and Child, 1933). Somewhat later, usually within 5 hours after section and before even the general form of the new hydranths is visible, such pieces show a distinct bipolar gradient, that is, reduction progresses from both cut ends toward the middle. In these cases reduction, like hydranth development, is usually somewhat more rapid distally than proximally, and the distal gradient extends somewhat farther from the distal end than the proximal gradient from the proximal end (Fig. 9). The bipolar reduction gradient persists during hydranth development except as it is altered locally by differentiation of hypostome and tentacles. The difference in time of development of distal and proximal hydranths and in length of distal and proximal reduction gradients are regarded as resulting from differences in condition at distal and proximal levels associated with the original basipetal gradient. In the distal region of the piece, there is an increase in rate of reduction in the hydranth region but no change in direction of the gradient, while in the proximal region a new gradient opposite in direction to the original develops. Apparently the development of this reversed gradient requires a certain length of time, and the presence of the distal gradient usually prevents its extension to the middle of the piece. In other words, the influence of the original gradient is still evident in the difference in time of hydranth development and in length of the reduction gradients at distal and proximal ends. If the original hy-

dranth remains on the piece for a sufficient length of time after proximal section and is kept in good condition by water circulation, hydranth development at the proximal end is inhibited in almost 100 per cent (Fig. 16), and some of the pieces give rise, after several days, to a basal structure and perisarc at the proximal end. In such morphologically unipolar pieces the reduction gradient is also unipolar and basipetal, except that some pieces show a limited area of reduction at the proximal end, which is shorter than a hydranth region and does not undergo extension distally (Fig. 13). This area probably represents an inhibited proximal gradient. In most, if not in all, cases it is clearly distinguishable from a hydranth gradient. If the hydranths are not maintained in good condition but are allowed to rest on the bottom and removed at different times after proximal section, the frequency of bipolar forms and of bipolar reduction gradients is greatly increased (Tables I, II). Apparently the dominance of the original hydranth depends not simply on its presence but on its physiological condition. The experimental data show that the dominance of the original hydranth prevents or partially inhibits the activation of the cells and the development of the new gradient, which appear to be essential factors in the formation of a new hydranth at the proximal end of the piece.

When the original hydranth is removed and proximal section is delayed (Fig. 21), hydranth development begins at the distal earlier than at the proximal end. With sufficient delay of proximal section, the developing distal hydranth becomes dominant and prevents hydranth development at the proximal end after section there. In these cases the reduction gradient is basipetal and unipolar except for the limited area of more rapid reduction at the proximal end in some pieces (Table III).

When distal and proximal section are made at the same time and the hydranth primordium at the distal end is removed by a second distal section made at different times after the first (Fig. 29), the developing proximal hydranth becomes dominant. With sufficient delay of the second distal section the dominance of the proximal hydranth may extend over the whole length of the piece and prevent development of a hydranth at the distal end in a high percentage of the pieces (Fig. 30). In such cases the reduction gradient is unipolar

but completely reversed in direction. Frequency of hydranth development at the distal end and of bipolar reduction gradients decreases as the period of delay of the second distal section increases.

The close parallelism between the unipolarity or bipolarity of reconstitution and of the reduction gradients constitutes evidence in support of the view that the reduction gradients are indicators of certain physiological characteristics of the longitudinal axis. The new polarity at the proximal end of the piece is indicated by a new gradient, which is evidently an expression of the activation of the cells following section and which precedes the appearance of the hydranth. From the higher levels of the gradient, the region of chief dominance develops.

The experiments of this paper agree with Child's earlier work (1927*a*, *b*, *c*; 1929*a*, 1932) in showing that the polarity of the *Corymorpha* stem is readily altered or reversed. They also show the rôle of the dominant region in maintaining polarity. When a dominant hydranth region is present, polarity is not usually reversed by the proximal section; but if the hydranth is removed, reversal of polarity in the proximal region almost invariably follows if proximal section is made at the same time. Dominance appears both in reconstitution and in the reduction gradients. It seems evident that the physiological differential of which the reduction gradient constitutes one aspect is an essential factor in hydranth development and dominance. That morphogenesis results from this differential appears beyond question, but how it results remains for future investigation to determine.

SUMMARY

1. In the experiments pieces 1.5–2 cm. in length from the distal naked-stem region of animals 4–5 cm. in length were used.

2. In pieces with distal and proximal section made at the same time, hydranths almost invariably develop at both ends, that at the proximal end slightly later than that at the distal.

3. When the original hydranth is present and in good condition, hydranth development at the proximal end is almost invariably inhibited and the pieces remain unipolar. If the hydranth is in poor condition, hydranth development at the proximal end occurs more frequently (bipolar forms).

4. With delay in removal of the original hydranth up to 72 hours after the time of proximal section, the frequency of unipolar forms increases from 2 to 92 per cent.

5. Delay of proximal section up to 48 hours after removal of the original hydranth increases the frequency of unipolar forms with hydranth at the distal end from 2 to 100 per cent.

6. The high unipolar frequency resulting from delay of either distal or proximal section is greatly decreased and bipolar frequency correspondingly increased by a second proximal or distal section made at the same time as the delayed section.

7. When distal and proximal section are made at the same time, and a second distal section, removing the distal hydranth primordium, is made with different periods of delay, the proximal hydranth becomes increasingly dominant. With 48 hours' delay of the second distal section the frequency of unipolar forms with hydranth at the proximal end and complete reversal of the polarity increases to 70 per cent.

8. A region where hydranth development will occur is usually directly visible 5–8 hours after section as a region of more rapid reduction of methylene blue with a gradient of decreasing rate of reduction extending from this region over a part or the whole length of the piece.

9. In pieces which develop as unipolar forms with hydranth at the distal end, the reduction gradient is, and remains, unipolar and basipetal except for later modifications in the hydranth region with differentiation of the hypostome, tentacles, etc., and the appearance in some pieces of a limited area of more rapid reduction at the extreme proximal end, which probably indicates an inhibited proximal gradient.

10. In pieces which develop as bipolar forms, the reduction gradient becomes bipolar; that is, reduction is most rapid at the two ends and decreases in rate toward the middle. In such cases reduction at the distal end is usually slightly more rapid than at the proximal end, and the distal reduction gradient is somewhat longer than the proximal. In these cases there is complete reversal in direction of the reduction gradient in the proximal region, as compared with the intact stem.

11. In pieces in which the hydranth region at the proximal end becomes dominant and hydranth development at the distal end is inhibited, the reduction gradient undergoes complete reversal in direction and is unipolar, the rate of reduction decreasing from the hydranth region at the proximal end.

12. The reduction gradients which arise in relation to regions of hydranth development become visible 10–16 hours before the form of the hydranth is distinguishable.

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A MORPHOLOGICAL PROOF OF THE EXISTENCE OF ZOOIDS IN EUPLANARIA DOROTOCEPHALA

(One figure)

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IN A number of papers on the axial gradients in planarians, Child and his collaborators have stated that asexual specimens of fissioning planarians, at least above a certain size, consist of two or more physiological units, so-called "zooids." This has been shown to be true particularly in *Euplanaria dorotocephala*. No attempt to review all these statements or to recapitulate the experimental data from which they were derived is made here. All the literature concerned has been recorded by Child in a later paper (1929). The first conclusions leading toward the establishment of the theory of zooids were based on a study of the regenerative power of different regions of the body (Child, 1906, 1910, and 1911).

Fission in *Euplanaria dorotocephala* occurs at a relatively fixed level some distance behind the pharynx. The prospective fission plane represents a physiological boundary line. The region posterior to it shows marked differences in its physiological characteristics from the region immediately anterior to it. Furthermore, each of the two main zooids may be divided into secondary, and even tertiary, zooids corresponding to the prospective individuals after the second and third fission. The existence of these physiological units has been shown by comparison of series of short pieces cut in sequence from the anterior to the posterior end of the full-grown animal. In such a series, the power of head regeneration decreases posteriorly up to the future fission plane, while the pieces posterior to it again are capable of forming normal heads.

Similar conclusions may be derived from experiments on the action of anesthetics on whole animals. The processes of disintegration, as studied by Child and others, likewise suggest the presence of several physiological units in adult asexual specimens.

All these deductions are based upon physiological experiments. No corresponding morphological differentiation has been found as yet. It should be of great value, for the support of the theory of zooids, to prove that the supposed physiological units can manifest themselves in anatomical features as well. In the following I shall report on observations which show that a morphological differentiation of the zooids can be induced under certain conditions. These data, though not strictly experimental, cannot fail to throw more light on the problems of the morphogenetic dynamics of the planarian body.

Thanks are due the Rockefeller Foundation, which enabled me to spend a year in the United States. I am likewise indebted to Dr. William A. Kepner and Dr. Ivey F. Lewis, who kindly extended to me the facilities of the Miller School of Biology, University of Virginia.

MATERIAL AND OBSERVATIONS

On October 11, 1931, numerous specimens of *Euplanaria dorotocephala* (Woodworth) were collected at Big Spring, near Kerr's Creek, Rockebridge County, Virginia. The animals were abundant under stones in the outlet of the big pond (water temperature, 19° C.). They were of different sizes, but all asexual, as far as one could judge from the external features. The animals were kept in two culture dishes—one at indoor temperature, the other in an electric refrigerator. The temperature in the laboratory varied considerably during the course of the observations, generally fluctuating between 20° and 30° C., but occasionally, for a short time, dropping to 17.5° C. or rising to 32.6° C. The culture in the refrigerator was maintained at a fairly constant temperature of 10°–12° C.

The animals were liberally fed on beef liver. In both cultures, fissions were observed, but not frequently. Unfortunately, no records of the number of fissions in each culture were made. The planarians increased in size; small specimens grew rarer. By November 30, many specimens of the room culture were found to possess genital pores indicating the differentiation of copulatory organs. At the beginning of January, 1932, the first cocoons were deposited in this culture.

Similar changes were observed in the refrigerator culture. Cocoons were laid during the period from February 22 to April 11, 1932.

After this, the animals ceased to lay egg capsules. All this time, but infrequently, fissions occurred, although it was difficult to decide whether they were normal fissions or pathological, autotomic, divisions. On May 24, when the culture was to be discontinued, the animals were subjected to a thorough inspection. There was a total of 71 specimens. Of these, 34 (47.9 per cent) were asexual, or, at least, without genital pores. These were, in general, smaller in size than the rest of the animals. Twenty specimens (28.2 per cent) proved to have one genital pore each. The remaining 17 animals

TABLE I

ABSOLUTE AND RELATIVE LENGTH OF THE PRE- AND POSTPHARYNGEAL REGIONS AND THE PHARYNX OF 3 SPECIMENS OF *Euplanaria dorotocephala* POSSESSING SUPERNUMERARY GENITAL PORES, AND OF ONE TYPICAL, SEXUALLY MATURE SPECIMEN

Measurements taken from preserved material.

	Prepharyngeal Region		Pharynx		Postpharyngeal Region		Total Length
	Mm.	Per Cent	Mm.	Per Cent	Mm.	Per Cent	Mm.
Specimen A.....	3.7	28.4	1.7	13.1	7.6	58.5	13.0
Specimen B.....	3.5	28.5	1.7	13.8	7.1	57.7	12.3
Specimen C.....	3.9	35.1	1.4	12.6	5.8	52.3	11.1
Normal specimen from the indoor culture.....	3.0	35.7	1.5	17.9	3.9	46.4	8.4

(23.9 per cent) each possessed from two to four genital pores. These specimens attained considerable size, up to 25 mm. in length, on average more than the ones with only one genital pore. The genital pores were situated one behind the other in the median line behind the mouth opening.

The differences between the body dimensions of adult specimens during the periods of asexual and sexual reproduction have already been dealt with by Hyman (1925, pp. 58 and 65), who also reviewed the data on this subject given by older authors. The specimens with supernumerary genital pores, as far as their external features are concerned, resemble fully grown asexual animals just before fission. The proportions of the prepharyngeal and postpharyngeal regions

and the pharynx of three specimens are shown in Table I and compared with those of a normal, sexually mature, specimen. We note that the specimens with additional genital pores have a comparatively longer postpharyngeal region than the normal specimen.

Three specimens with supernumerary pores (specimens A, B, and C, the same as those referred to in Table I) were examined anatomically. For the normal structure of the genital organs of *Euplanaria dorotocephala* Hyman's paper (1925) may be quoted. It gives a good description of the anatomy of the species and corrects a few misstatements made by previous authors.

The study of the three specimens revealed the following facts:

The testes are fully developed, in the normal position near the ventral side of the body, extending along almost the entire body. The ovaries (germaria) are large and, generally, form lobes. Parts of them may even detach from the main ovary and lie separated in the parenchyma.[1] The vitellaria, or yolk glands, are well differentiated in all three specimens.

In a way similar to that of the genital pores, the inner parts of the copulatory apparatus are likewise multiplied. Figure 1, *A*, *B*, and *C*, shows diagrams of longitudinal sections through the reproductive apparatus of the three specimens, A, B, and C.

Each genital pore leads into a cavity, the genital atrium. The size and shape of this cavity depends, mainly, on the completeness of the remaining organs, particularly of the penis. Where the penis is well developed (e.g., A I and II), the atrium is almost as in normal specimens. On the other hand, an atrium is present even there where no penis is differentiated (A III, IV; C III). In this case, it is approximately tube- or sac-shaped, with a somewhat irregular outline. The most rudimentary state of a copulatory apparatus is that consisting of a simple cavity in the parenchyma, with even the genital pore missing (B IV). The cavity corresponds, in the main, to a reduced genital atrium. Only its dorsal part, differing from the remaining portion by the histology of the epithelial lining, must be considered homologous to the end portion of a bursa stalk. Wherever an atrial

[1] Similar supernumerary ovaries, though arranged more regularly, have been occasionally observed by Beauchamp (1932, p. 300) in a European species, *Fonticola vitta* (Dugès).

cavity appears, special glands are developed, which open through the epithelium of the cavity. Their granular secretions stain orange or pink after treatment with haematoxylin and erythrosin. They

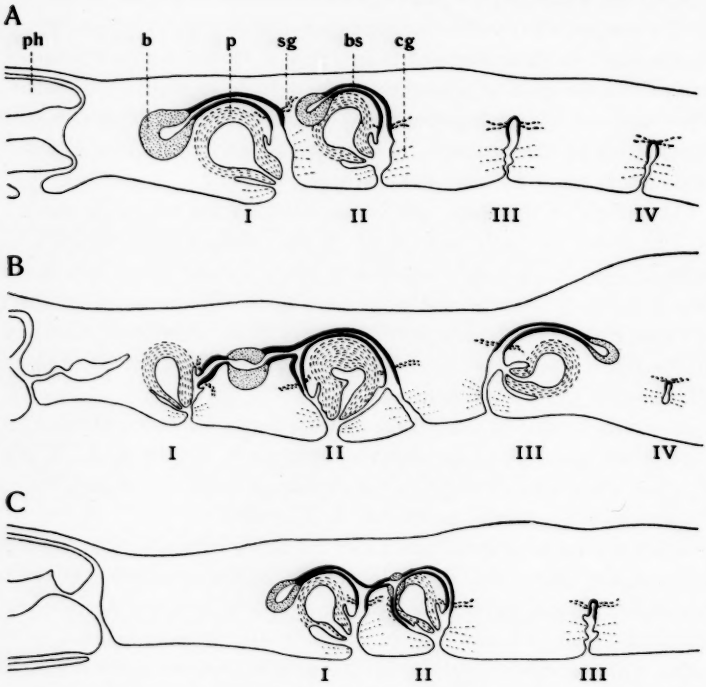

FIG. 1.—*Euplanaria dorotocephala*, diagrams of longitudinal sections through the postpharyngeal regions of three specimens (*A*, *B*, and *C*) possessing supernumerary copulatory apparatus. ×27.

I, *II*, *III*, *IV*, first, second, third, and fourth apparatus; *b*, bursa; *bs*, bursa stalk; *cg*, cement glands; *p*, penis; *ph*, pharynx; *sg*, shell glands.

are the cement glands.[2] A particular feature appears in the second copulatory apparatus of the specimen B: two genital pores leading into the same atrial cavity.

The penis, where developed, likewise shows various degrees of dif-

[2] Hyman (1925, p. 70) considered them to be shell-forming glands. In a later paper (1929, p. 409), she called them, correctly, cement glands.

ferentiation. These concern chiefly the size and shape, while the histological structure is always that of a fully developed organ. A spherical bulb, imbedded in the parenchyma, and the penis itself, i.e., a papilla projecting into the atrium, can be distinguished in all cases. The seminal vesicle is usually spherical, swollen, and filled with glandular secretions. The seminal ducts (vasa deferentia), where present, open into horn-shaped outgrowths of the lumen, one on either side. The main axis of the penis lies, in most cases (A I, II; B I; C I, II), in the normal direction, i.e., with the penis pointing posteriorly and ventrally. The organ B II, however, is oriented dorsoventrally, and the penis B III shows, like the entire complex of organs to which it belongs, an inverse polarity, with the papilla pointing anteriorly.

As to the bursa copulatrix and its outlet into the atrium, the bursa stalk, great irregularities occur in several instances. The bursa is rather small, sac-shaped, and lined with a thick epithelium, so that the lumen is restricted to a narrow cavity. Each copulatory apparatus equipped with a penis is connected with at least one bursa. In about one-half the number of cases (A I, II; B III), the connection is a typical one, the arched bursa stalk lying above the penis. In accordance with the inverse polarity of the entire apparatus, the bursa stalk B III proceeds from the atrium posteriorly instead of anteriorly. In several cases, additional connections between the atria and the bursae occur. A second bursa stalk may start from the atrium and lead either directly into the bursa (C II) or into the typical bursa stalk (B II). A supernumerary bursa stalk may even connect two neighboring copulatory apparatus (C I and II). In B I, a bursa stalk starts from the atrium in the typical place; but instead of curving anteriorly, it proceeds caudally to open into the bursa of the second apparatus. No bursa is differentiated in B I; an irregularly shaped cavity in the parenchyma, occurring in the typical site of the bursa, should perhaps be considered homologous to this organ. It shows, however, no bursa-like histological structure and opens into the pharyngeal chamber.

In cases where no bursa is developed, a rudimentary bursa stalk nevertheless is differentiated (A III, IV; B IV; C III). It appears as a short, blindly closed outgrowth of the atrium and corresponds

to the posterior portion of a typical bursa stalk. It can easily be recognized by the openings of gland ducts with granular, strongly eosinophilic secretion (shell glands).

The copulatory apparatus lie, in general, at a greater distance from the pharynx than in normal specimens. This is due to a larger mass of parenchyma intervening between the posterior wall of the pharyngeal chamber and the first complex of copulatory organs. As a rule, the anterior apparatus are more complete than the posterior ones. The best developed is either the first (A) or the second (B); or the first two are of about equal size (C). This difference in development concerns the size, shape, and completeness only. The histological differentiation, however, is complete in all organs, even in those instances where only a rudimentary cavity is developed and neither a penis nor a bursa is present.

The connections between the genital glands and the copulatory organs are of particular interest. Unfortunately, the examination of the male gonoducts (vasa deferentia) is, technically, rather difficult. These canals show up very clearly in the region of the pharynx, where they are swollen with sperm. In normal animals, the connection between these enlarged tubes (the "false seminal vesicles") and the male copulatory organs can be easily established. On the other hand, the ducts leading from the testes to the false seminal vesicles are very delicate structures and cannot be traced, in their full extension, in the preparations. Usually, only parts of this system are to be found in the slides. The same applies, in our case, to eventual connections between the false seminal vesicles and incompletely developed copulatory organs. With the inevitable reservation regarding accuracy of observation, the following can be said of these connections:

Specimen A.—Penis I is, on both sides, connected with the vasa deferentia. From the seminal vesicle II, a canal starts on the left side, but ends blindly within the musculature of the penis bulb. On the right, a vas deferens appears to be lacking entirely.

Specimen B.—The organs I and II are connected with the male ducts on both sides. In penis III, the left side lacks a connection, while on the right there is a short, blind extension of the seminal vesicle.

Specimen C.—Apparatus I has typical connections, II shows a tube leading off the seminal vesicle on either side, but apparently ending in the parenchyma.

The female gonoducts or oviducts, typically one pair proceeding from the ovaries posteriorly along the ventral nerve trunks, show fewer irregularities in our material. On either side, the oviduct empties into one of the bursa stalks, right above the place where the shell glands open into this. The copulatory apparatus with which they connect is either the first (C) or the second (B); or, finally, the oviduct of the left side opens into the first, the right one into the second bursa stalk (A). All the remaining apparatus lack connections with the ovaries.

DISCUSSION AND CONCLUSIONS

Supernumerary structures in the main axis of the body and with the same (homopolar) orientation towards this axis, have been but rarely observed in planarians. Wilhelmi (1909, p. 67 and pl. 10, fig. 37) reports of a specimen of *Fonticola morgani* (Stevens and Boring) possessing two pharynges one behind the other. A similar abnormality I observed in *Euplanaria maculata* (Leidy). Then, one case of a partially doubled copulatory apparatus has been seen in the European species, *Polycelis tenuis* Ijima (Kenk, 1927). The causes that induced the formation of these double structures cannot be indicated with any reasonable degree of probability, since all experimental evidence with regard to them is lacking. Perhaps these structures are due to mechanical lesions of some kind. In any case, their sporadic occurrence indicates that they are abnormalities apparently brought about by accidental causes.

On the other hand, a regular formation of supernumerary organs has been noted in a few fissioning planarians immediately before asexual reproduction. Kennel (1883 and 1888, pp. 468–72) reports that in "*Planaria fissipara*" of Trinidad the second zooid before its separation forms a new pharynx, a pair of eyes, and new cephalic ganglia. *Euplanaria paramensis* (Fuhrmann), which divides at a level anterior to the pharynx, regenerates a new pharynx in the anterior zooid before fission (Fuhrmann, 1914, p. 801). In these two instances, the formation of the double organs is, apparently, to be considered as advance regeneration of organs before asexual reproduc-

tion. It corresponds perfectly to the well-known, regular formation of chains of zooids in many rhabdocoels (*Catenulidae* and *Microstomidae*).

There appears to be a certain relationship between the phenomena of premature regeneration of organs and the development of a great number of pharynges in the so-called "polypharyngeal" planarians (Mrázek, 1904, pp. 33–40; and 1914, pp. 265–76). Several European species of the genus *Crenobia*, e.g., *C. montenigrina* (Mrázek), and the American "*Phagocata*" *gracilis* (Haldeman) possess numerous (three to thirty-five) pharynges instead of only one, as is typical. The pharynges are not arranged in a single series, but are attached to the lateral walls of the primary pharyngeal chamber and alternate more or less regularly on the right and left side. They can be protruded through the common mouth opening. There is no doubt that the polypharyngeal species originated from monopharyngeal ones. The *Crenobia* forms evidently arose from *C. alpina* (Dana), and *Phagocata gracilis* has been claimed to be derived from *Fonticola morgani*.[3] Mrázek thinks that the number of pharynges increased in much the same way as the second pharynx is formed in *Planaria fissipara*, viz., by regeneration of the organ previous to fission; the fission itself, however, did not take place—it was suppressed. If this process be repeated, a great number of pharynges must result. Actually, we see that the number of pharynges increases as the animal grows larger. Primarily, the new pharynx ought to lie behind the old ones, on the assumption that the hypothetic fission plane is postpharyngeal. But since the size of the body is limited and the pharynges develop close to each other, they arrange themselves biserially. Mrázek's theory has been criticized, particularly by Wilhelmi (1909, pp. 69–72). Nevertheless, it accords better with the facts observed than Wilhelmi's hypothesis, which claims a traumatic origin of the polypharyngia.

Moreover, analogous multiplications of organs might be sought for in other taxonomic groups. Among the *Platyhelminthes*, the *Cestoda* give an outstanding example of highly differentiated zooids

[3] This appears to me somewhat doubtful, since both species differ considerably with regard to their reproductive systems.

(proglottids). Here the interpretation is essentially the same as that given by Mrázek for polypharyngeal planarians.

If we compare all these cases of supernumerary axial organs with our observations on *Euplanaria dorotocephala*, it appears probable that there exists a great similarity between the formation of several copulatory apparatus in our material and the polypharyngia of certain species of planarians. Furthermore, the suppression of fission, that has been made responsible for the polypharyngia, can almost be proved in our case.

In *Euplanaria dorotocephala*, asexual reproduction by fission occurs very regularly. Besides this, but apparently not at the same time, sexual propagation takes place under certain conditions. Sexually mature animals have been but very rarely observed in the field (*cf.* Hyman, 1925, pp. 64–65), oftener in laboratory cultures. The precise conditions that induce the animals to develop sex organs are not known as yet. There seem to exist, as in several other species of fissioning fresh-water triclads, different physiological races of *E. dorotocephala*, in which the tendency to become mature differs considerably. Hyman's observations indicate that the normal period of sexual reproduction is the early spring. In laboratory cultures, usually only a small percentage of the animals form reproductive organs. While I had my collections in Virginia, I noted, in the natural habitats, only asexual animals. Raised in laboratory cultures, specimens from certain localities became mature; those from other localities failed to do so. In the material from Big Spring, to which the present report refers, the majority of the animals attained maturity.

Supernumerary copulatory apparatus occurred only in the culture that was kept at a constant low temperature. Low temperature is known materially to decrease the rate of asexual reproduction in planarians and, in *Euplanaria maculata*, can suppress fission entirely for several months.[4] Nevertheless, the animals grow large if fed well. In our case, the process was apparently the following: The animals, which were collected in an asexual state, consisted, at least the larger ones, of two or more physiological zooids. Because of the low temperature, a separation of these zooids did not take place—at least

[4] Unpublished observations.

not in all specimens. Actually, fissions were observed very rarely. The animals increased in size and, under the changed conditions which cannot be analyzed in detail, developed sex organs. Each zooid formed its own, more or less complete, copulatory apparatus. These must, therefore, lie one behind the other. The large number of specimens that show this abnormality (17 in the culture of 71) excludes a mere accidental (e.g., traumatic) origin of the supernumerary organs.

The development of copulatory organs in the posterior zooids indicates that the zooids correspond to more or less independent postpharyngeal parts of the body. They do not, previous to separation, differentiate any organs characteristic of the anterior end. Only after detachment from the anterior zooids, are new heads and pharynges formed. As long as they are not separated, their morphogenetic powers are those of the postpharyngeal region.

Like other cases of atypical formation of organs, our material furnishes evidence of the action of several factors that are involved in the differentiation of the organs. This refers particularly to the question of how far the formation of the copulatory apparatus, or of parts thereof, is controlled by the genital glands and how far these organs may differentiate independently.

We know from a number of papers (cf. the quotations in Kenk, 1923) that the development of the copulatory organs begins without direct (morphological) connection with the gonads. Nevertheless, the stimulus for their differentiation seems to start from the gonads. This is indicated particularly by the investigations of Vandel (1920 and 1922, pp. 471–72), which show the probable existence of some kind of sex hormone. The morphological connection between the atrial organs and the gonads, by means of the gonoducts (vasa deferentia and oviducts) develops only much later. In our case, the majority of the organs remained permanently independent (morphologically) of the gonads. The incomplete parts of the apparatus cannot be considered as being at earlier stages of development, as one might be tempted to suppose. All of them, including the most rudimentary ones, had reached the final stage of histological differentiation. The animals had already stopped laying egg capsules, and a

further development of the reproductive system could not be assumed.

As to the vasa deferentia, our observations confirm the data of previous authors. They develop in two independent portions: one portion growing out of the testes, the other out of the male copulatory organ.

Oviducts are always only one pair, as is typical. They open, on either side, into one of the bursa stalks, both into the same or into two different copulatory apparatus. The rest of the bursa stalks lack oviducts. This is somewhat surprising. We know that in some planarians the end portion of the oviduct grows from the atrium and only secondarily joins the lateral oviducts which proceed from the ovaries. Were this the case here, traces, at least, of the atrial outgrowths ought to be seen in the well-developed atrial organs. There seems to be a fundamental difference in the two main types of planarians, as indicated by Meixner (1928, pp. 578–80): one type in which both oviducts fuse to form a "common oviduct" which enters the genital atrium and is connected with shell glands (i.e., the majority of the fresh-water triclads); and another (comprising the genera *Euplanaria* and *Curtisia*) in which the oviducts open more or less independently into the bursa stalk and the shell glands likewise empty into this. In the first type, at least the common oviduct originates as an outgrowth of the atrium (e.g., in *Polycelis tenuis* Ijima; see Böhmig, 1913, and Kenk, 1927). In the second, the posterior or atrial *anlage* may not develop.[5]

It is interesting that in all copulatory apparatus shell glands are present. This is the case even in the most rudimentary apparatus where penis and bursa are missing (A III, IV; B IV; C III). Where there is no typical bursa stalk, the glands open into a very short upper extension of the atrial cavity. For this reason, the extension was considered to be a rudimentary bursa stalk. There is, however,

[5] In a previous paper (1923), I expressed a different opinion on the development of the oviduct in *Euplanaria lugubris* O. Schmidt (*"Planaria polychroa"*), viz., that the posterior parts of the oviducts start developing from the bursa stalk. Since the early stages of these ducts are very thin and cannot easily be traced in serial sections, their possible connection with the anterior parts of the oviducts may have been overlooked.

another interpretation of the outgrowth possible: it may be a common oviduct formed from the wall of the atrium. In normal specimens, this common oviduct does not show up separately, as it is fused with the bursa stalk. If this be true, the difference between the two types of planarians, as regards the openings of the shell glands, would not really exist.

It is not possible to interpret the many irregularities that occur in the number and position of the bursa stalks. We do not know much about the factors that control their development.

The general orientation of the copulatory apparatus with regard to the polarity of the body is usually typical. This is best seen in the position of the penis, which points toward the posterior end of the body. There are, however, two exceptions to this rule, both in the same specimen, B. Here penis II has a dorsoventral axis; and apparatus III, in all its parts (penis, genital pore, bursa, and bursa stalk) shows inverse polarity. It cannot be determined whether this conforms with the polarity of the entire third zooid, since no other polar organs exist in this region. Unfortunately, the specimen had not been closer examined while alive; the direction of the ciliary movement at the corresponding level of the body might have indicated the polarity of this region.

It would be of great interest to investigate the process of the formation of supernumerary reproductive organs in a more accurate experimental way. My stay at the University of Virginia, where the observations were made, did not suffice for a repetition of the experiment on a larger scale. I believe that it is essential to choose the right physiological race for investigation. It is certain, as was pointed out above, that animals from different localities show different tendencies for sexual reproduction. The animals must be such that under laboratory conditions they will readily develop sex organs. Moreover, the season at which the experiment is started may be of decisive importance for the result. The animals at the beginning of the experiment must be in a period of lively asexual propagation.

A thorough experimental study of the phenomena described would, in my opinion, reveal further important facts concerning the morphogenetic correlations of the planarian body.

SUMMARY

1. In a culture of *Euplanaria dorotocephala* from Big Spring, near Kerr's Creek, Virginia, kept at low temperature for several months, many animals developed supernumerary copulatory apparatus lying one behind the other. The reproductive systems of three specimens were examined anatomically.

2. The formation of these organs was due to the presence of several zooids at the time when the differentiation of the reproductive system took place. The low temperature had prevented the prior separation of the zooids.

3. The copulatory apparatus are not all connected with the genital glands. They are formed independently of the gonads and only secondarily connect with them by the genital ducts. The vasa deferentia originate in two sections, the one starting from the testes, the other from the seminal vesicle. The oviducts appear to be formed in one pair starting from the ovaries.

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A DIETARY FACTOR WHICH IMPARTS TO CERTAIN MAMMALIAN TISSUES A QUALITY NECESSARY FOR THE CORRECT NUTRITION OF PLANARIAN WORMS[1]

(Seven figures and one plate)

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THE planarian worm, a carnivorous animal, has proved itself to be a useful instrument for the demonstration of changes in the composition of mammalian tissues resulting from altered conditions in the animals furnishing the tissues (Bahrs, 1931).

During an investigation on the effects produced in animal tissues by the presence or absence of the recognized vitamins in the diets of the animals furnishing the tissues, it was found in general that, no matter what the vitamin under consideration, a diet of liver or heart from these animals caused the development of dietary disease in the planarian worms. The only heart and liver tissues free from harmful effects were derived from guinea pigs which had been fed fresh green kale.

The syndrome of the disease was as follows: When gliding freely, planaria have a beautifully smooth outline; and the first sign of trouble was a roughening of the smooth border. This was followed by a deepening in color of the whole worm; and as the disease advanced, the surface of the worms shriveled and became almost black. Irregular lumps developed, which in the worst cases became so large as to bend the worms into abnormal shapes and made it impossible for them to move. Although the worms showing this pathological condition continued to eat after the onset of the trouble, the result of the disease was shown early by a decrease in growth. The accompanying series of drawings gives some idea of the gross appearance

[1] Aided by a grant from the General Research Council of the Oregon State System of Higher Education.

of the disease (see Fig. 1). This syndrome is essentially different from the one observed by us in connection with the administration to planaria of liver and egg white in graded amounts (Wulzen and Bahrs, 1931). This was characterized by the appearance of clear blisters which, rupturing, led to the disintegration of the worms.

The present experiments were conducted in the following manner: Guinea pigs were fed a basal diet designed to provide the necessary

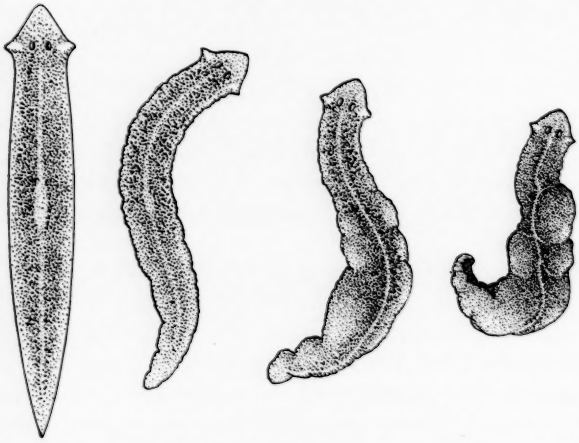

FIG. 1.—Planaria showing development of the dietary disease recorded in these experiments. Note increasing irregularity of lateral borders and the appearance of lumps which greatly deform the entire worm. The worm also shrivels and darkens.

and desirable food constituents in the correct proportions for maintaining the animals in optimum condition. There was one omission: the basal diet lacked vitamin C. This was added in ample amounts for each experimental group in the form of the supplements: orange juice, tomato juice, fresh grass, or fresh kale. The guinea pigs were kept on this diet for a month and at that time experimental groups of planarian worms were prepared. (The worm used was probably *Planaria agilis*.) These worms were newly regenerated from tails cut from our stock planaria. The worms were carefully measured as they glided freely over polar co-ordinate paper, and were distributed into groups of thirty. In almost all experimental groups every worm had

the same length measurement. Worms 6, 7, and 8 mm. in length were used. The worms were kept in finger bowls containing river water sterilized by heat. All experimental groups were maintained at a constant temperature, 23°–24° C. The worms were fed twice a week on tissues of freshly killed experimental guinea pigs. The tissues used were spleen, lung, liver, heart, and kidney cortex. Bits of these tissues were mashed until they were in the form of a paste, and were then fed to the worms as a pellet. The food was left before the worms for at least 4 hours and was then removed and the worms cleaned and given fresh water. They always went to the tissues quickly and ate until distended. Measurements of the worms were taken every week, and the total gain in length of each experimental group has been plotted on the charts.

A preliminary experiment was arranged in which one group of experimental guinea pigs was fed a basal diet of rolled barley, 60 per cent; alfalfa meal, 40 per cent; salt *ad lib.*, with the addition of fresh grass, 10 g. per animal per day. Another group of guinea pigs was fed the same basal diet, and in addition each animal received by means of a medicine dropper 10 cc. of canned tomato juice per day. After the feeding had continued for a month, groups of planarian worms were prepared. The worms were fed twice a week on spleen, lung, liver, or heart tissue from freshly killed guinea pigs, representing both experimental groups, and the feeding continued for a month. The results are shown graphically in Figure 2 and are outlined below.

Spleen.—All worms fed spleen remained healthy throughout the 4 weeks of the experiment. Spleen showed high growth-promoting power, though (grass) spleen[2]—length gain, 226 mm., 102 per cent ($M = 14.289 \pm .194$)—gave a noticeably better growth than (tomato) spleen—length gain, 163 mm., 74 per cent ($M = 12.785 \pm .183$). All worms were healthy at the end of the experiment.

Lung.—On the lung diets growth was fair, and all worms remained healthy. (Grass) lung gave a length gain in the planarian worms of

[2] The term in parenthesis before the name of a tissue designates the supplement which had been fed to the guinea pig supplying the tissue. For example, "(kale) spleen" means spleen derived from a guinea pig fed kale as a supplement to a scurvy-producing basal diet. "(Non-kale) liver" means liver derived from an animal fed the scurvy-producing basal diet without any supplement.

76 mm., 40 per cent ($M = 8.896 \pm .145$), while (tomato) lung produced a gain of 84 mm., 44 per cent ($M = 9.160 \pm .130$).

Liver.—The two diets produced livers of profoundly different quality as far as the worms were concerned. (Grass) liver had ex-

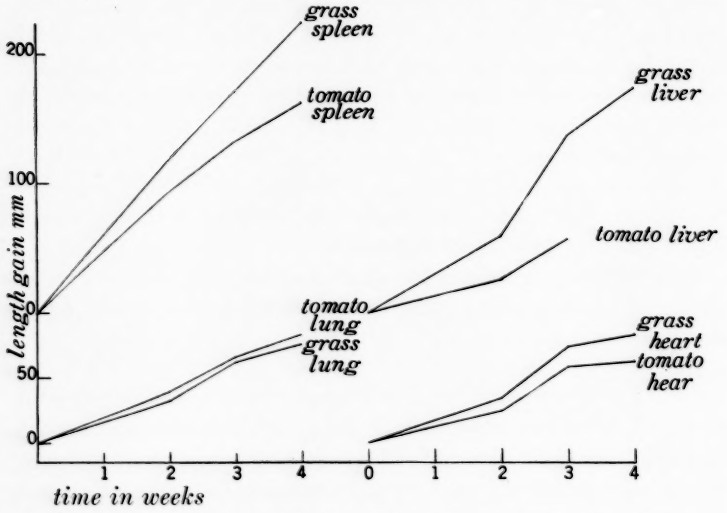

FIG. 2.—Each curve represents length gain in millimeters of thirty planarian worms fed the tissue diet specified on the curve.

Diets of guinea pigs furnishing tissues:

Basal-grass diet, yielding (grass) tissues:

Ground barley	40 per cent
Skim milk powder, baked	26 per cent
Clarified butter	10 per cent
$CaCO_3$	3 per cent
NaCl	1 per cent

Fresh grass, 10 g. per animal per day

Basal-tomato diet, yielding (tomato) tissues:

Same as above, no grass
Canned tomato juice, 10 cc. per animal per day

cellent growth-promoting power, and at the end of the experiment the worms were entirely healthy. The length gain was 173 mm., 91 per cent ($M = 13.103 \pm .221$). In contrast to this, when worms were fed (tomato) liver they very quickly became diseased; and at the end of 4 weeks it was impossible to get measurements of the group

because the damage was universal and very severe. Growth was, of course, slight.

Heart.—The heart tissue diets had an almost equal, low growth-promoting power for the worms. (Grass) heart gave a length gain of 83 mm., 34 per cent ($M = 10.766 \pm .143$), while (tomato) heart gave a length gain of 62 mm., 25 per cent ($M = 10.071 \pm .172$). The pathological effect of heart tissue, however, was in striking contrast. The worms fed (grass) heart remained entirely healthy; while in the group of worms fed (tomato) heart, 50 per cent were found more or less affected by dietary disease at the conclusion of the experiment.

Summary.—It is evident that canned tomato juice cannot replace fresh grass as a supplement to the selected basal diet if guinea-pig heart and liver tissue are to result which will not give rise to dietary disease in planarian worms. Since the basal diet contained dried alfalfa, we may also infer that dried alfalfa cannot act as a source of the substance which must be present in the diet of guinea pigs if liver and heart tissue are to be produced which will maintain planarian worms in a healthy condition.

To continue the investigation, fresh kale, orange juice, fresh tomato juice, and canned tomato juice were used as supplements to the following basal diet: ground barley, 40 per cent; milk powder baked to eliminate vitamin C, 26 per cent; bran, 20 per cent; clarified butter, 10 per cent; calcium carbonate, 3 per cent; sodium chloride, 1 per cent. Five experimental groups of young guinea pigs were prepared. One group was left on the basal diet alone as a control. This was only as a test of the basal diet and when these guinea pigs developed undoubted scurvy the group was discontinued. Another group received, in addition to the basal diet, 10 g. of fresh kale per animal daily. The animals all ate the kale as soon as it was placed in the cage, and there was no question that each received its share. The three remaining groups received, respectively, orange juice, fresh tomato juice, and canned tomato juice as supplements. In order that all individuals might have an equal share, 10 cc. of the supplements were administered to each animal daily with a medicine dropper.

The feeding of the guinea pigs was continued for a month, and then experimental groups of planarian worms were started on the

tissue diets. Spleen, lung, liver, and heart were used; and the experiment was continued for 6 weeks. After the series of assays had been under way for a week, it was decided to add kidney cortex to the list of tissues; and the experiment on this tissue was continued for 5 weeks. The results were as follows:

Spleen.—Throughout the 6 weeks of the experiment, the worms grew very rapidly on all four varieties of spleen tissue, no matter what the supplement; and all the worms were in excellent condition at the conclusion of the experiment. (Kale) spleen gave a total length gain of 314 mm., 149 per cent ($M = 17.481 \pm .176$); (orange) spleen, 345 mm., 169 per cent ($M = 18.678 \pm .247$); (fresh tomato) spleen, 317 mm., 150 per cent ($M = 17.566 \pm .167$); (canned tomato) spleen, 373 mm., 177 per cent ($M = 19.444 \pm .169$). (See Fig. 3.)

Lung.—The results following the feeding of lung tissue resembled those obtained from the feeding of spleen. No matter what supplement had been fed the guinea pigs producing the lung tissue, the tissue exhibited steady and very good growth-promoting power for the worms, and all worms were healthy at the close of the experiment. The growth was somewhat less than that on spleen. (Kale) lung gave a total length gain of 322 mm., 134 per cent ($M = 18.750 \pm .153$); (orange) lung, 308 mm., 128 per cent ($M = 18.285 \pm .165$); (fresh tomato) lung, 322 mm., 134 per cent ($M = 18.733 \pm .147$); (canned tomato) lung, 327 mm., 136 per cent ($M = 18.900 \pm .182$). (See Fig. 4.)

Liver.—The four supplements, when fed to guinea pigs, yielded livers all of which had excellent growth-promoting power for the planarian worms during the first 3 weeks of the experiment, although almost at once kale had separated from the rest in its superior ability to produce liver with high growth-promoting power. Through the 6 weeks of the experiment the worms fed liver derived from guinea pigs receiving kale supplement grew with exceeding rapidity and were exceptionally large and well nourished when the experiment terminated. The length gain of the group was 450 mm., 250 per cent ($M = 21.000 \pm .162$). In striking contrast was the effect produced on the worms by the livers derived from guinea pigs receiving fresh and canned tomato juices as supplements. At the end of the third week the worms fed these livers showed definite signs of disease; and al-

though they continued growing through the fifth week, they were becoming more and more seriously affected, until at the end of the

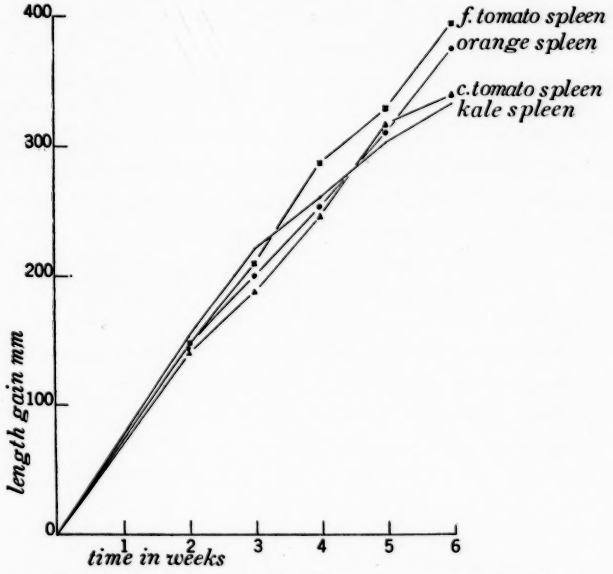

FIG. 3.—Each curve represents length gain in millimeters of thirty planarian worms fed the tissue diet specified on the curve.

Diets of guinea pigs furnishing tissues:

Basal diet:

Ground barley	40 per cent
Skim milk powder, baked	26 per cent
Clarified butter	10 per cent
$CaCO_3$	3 per cent
NaCl	1 per cent

Basal-kale diet, yielding (kale) spleen:
Same as above
Kale, 10 g. per animal per day

Basal-orange diet, yielding (orange) spleen:
Same as above
Orange juice, 10 cc. per animal per day

Basal–fresh tomato diet, yielding (fresh tomato) spleen:
Same as above
Fresh tomato juice, 10 cc. per animal per day

Basal–canned tomato diet, yielding (canned tomato) spleen:
Same as above
Canned tomato juice, 10 cc. per animal per day

sixth week the disease was universal and very severe. (Fresh tomato) liver gave a total length gain of 235 mm., 130 per cent ($M = 14.074 \pm$

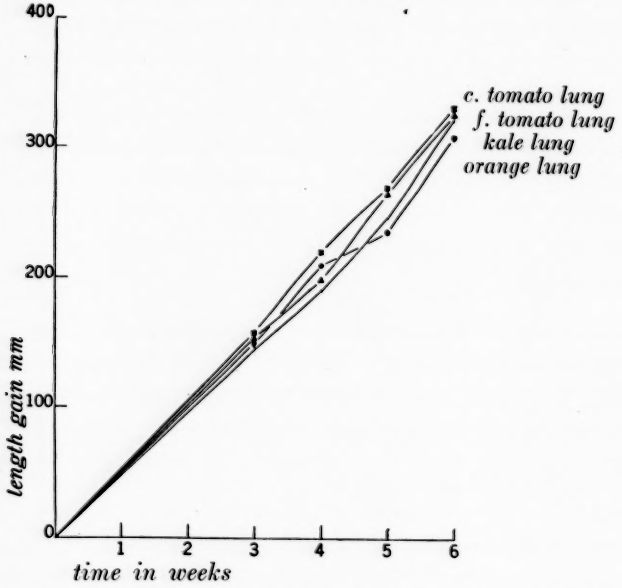

FIG. 4.—Each curve represents length gain in millimeters of thirty planarian worms fed the tissue diet specified on the curve.

Diets of guinea pigs furnishing tissues:

Basal diet:

Ground barley	40 per cent
Skim milk powder, baked	26 per cent
Clarified butter	10 per cent
$CaCO_3$	3 per cent
NaCl	1 per cent

Basal-kale diet, yielding (kale) lung:
Same as above
Kale, 10 g. per animal per day

Basal-orange diet, yielding (orange) lung:
Same as above
Orange juice, 10 cc. per animal per day

Basal–fresh tomato diet, yielding (fresh tomato) lung:
Same as above
Fresh tomato juice, 10 cc. per animal per day

Basal–canned tomato diet, yielding (canned tomato) lung:
Same as above
Canned tomato juice, 10 cc. per animal per day

.336); and (canned tomato) liver, 247 mm., 137 per cent ($M = 14.482 \pm .312$). Orange juice as a supplement produced guinea-pig livers which were not quite so damaging in their effect. The worms gained somewhat more rapidly than they did on the livers of guinea pigs receiving tomato juice; and the disease, though widespread at the conclusion of the experiment, was not so devastating. (Orange) liver gave a total length gain of 367 mm., 203 per cent ($M = 18.517 \pm .271$). All the worms with the exception of three were affected by the dietary disease, but only in six was the damage severe. (See Fig. 5.)

Heart.—All groups of worms fed heart showed a fair rate of growth throughout the experiment, and those receiving (kale) heart remained entirely normal. The length gains of the planarian worm groups were 236 mm., 112 per cent ($M = 14.896 \pm .177$) for (kale) heart; 225 mm., 107 per cent ($M = 14.500 \pm .179$) for (orange) heart; 194 mm., 92 per cent ($M = 13.482 \pm .157$) for (fresh tomato) heart; and 192 mm., 91 per cent ($M = 13.400 \pm .202$) for (canned tomato) heart. The group of planarian worms fed (orange) heart showed eleven worms affected by dietary disease at the conclusion of the experiment. In the group fed (fresh tomato) heart, fourteen worms were affected; and in the group fed (canned tomato) heart, seventeen worms were affected, most of them severely. (See Fig. 6.)

Kidney.—The worms fed (kale) kidney grew steadily; and at the end of the experiment the length gain was 201 mm., 111 per cent ($M = 13.137 \pm .193$). They remained normal but seemed slow in movement. Kidneys from guinea pigs receiving the orange and tomato juice supplements had very low growth-promoting power and proved to be strongly pathogenic for the worms. At the conclusion of the experiment they were all too diseased to be measured. (See Fig 7 and Plate I.)

DISCUSSION

Throughout these experiments, the guinea pigs which were killed to supply tissue for the diet of the worms were all healthy in appearance and were growing well, as their weight records showed. No matter whether orange juice, tomato juice, or kale had been given them, at autopsy the guinea pigs displayed no overt signs of disease. The control group of guinea pigs on the same basal diet but without

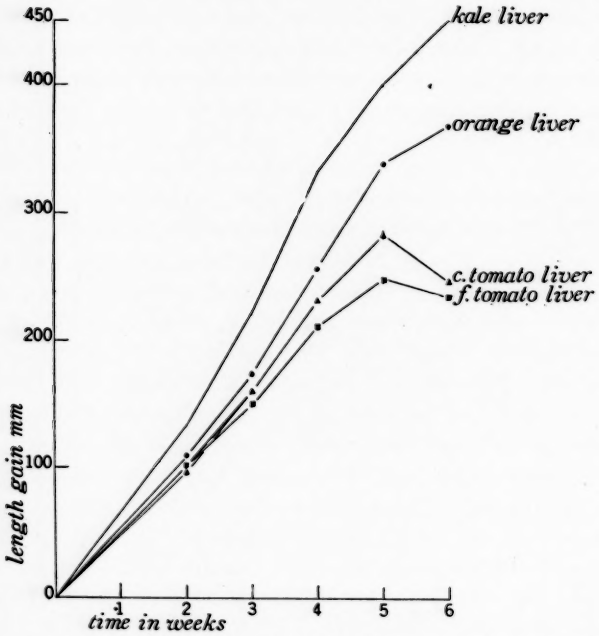

FIG. 5.—Each curve represents length gain in millimeters of thirty planarian worms fed the tissue diet specified on the curve.

Diets of guinea pigs furnishing tissues:

Basal diet:

Ground barley	40 per cent
Skim milk powder, baked	26 per cent
Clarified butter	10 per cent
$CaCO_3$	3 per cent
NaCl	1 per cent

Basal-kale diet, yielding (kale) liver:
Same as above
Kale, 10 g. per animal per day

Basal-orange diet, yielding (orange) liver:
Same as above
Orange juice, 10 cc. per animal per day

Basal–fresh tomato diet, yielding (fresh tomato) liver:
Same as above
Fresh tomato juice, 10 cc. per animal per day

Basal–canned tomato diet, yielding (canned tomato) liver:
Same as above
Canned tomato juice, 10 cc. per animal per day

any of these supplements very early in the experiment developed scurvy and was discontinued.

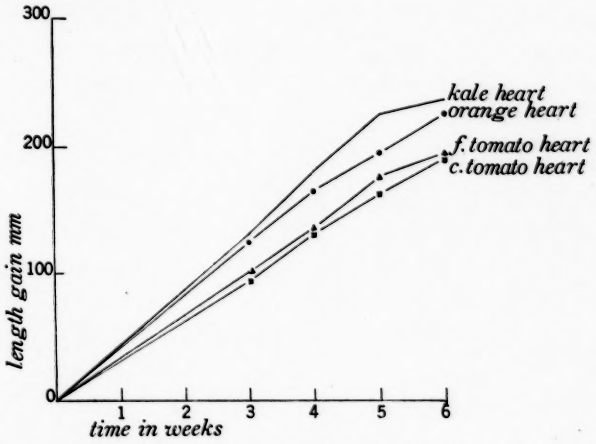

FIG. 6.—Each curve represents length gain in millimeters of thirty planarian worms fed the tissue diet specified on the curve.

Diets of guinea pigs furnishing tissues:

Basal diet:

Ground barley	40 per cent
Skim milk powder, baked	26 per cent
Clarified butter	10 per cent
$CaCO_3$	3 per cent
NaCl	1 per cent

Basal-kale diet, yielding (kale) heart:
Basal diet
Kale, 10 g. per animal per day

Basal-orange diet, yielding (orange) heart:
Basal diet
Orange juice, 10 cc. per animal per day

Basal–fresh tomato diet, yielding (fresh tomato) heart:
Basal diet
Fresh tomato juice, 10 cc. per animal per day

Basal–canned tomato diet, yielding (canned tomato) heart:
Basal diet
Canned tomato juice, 10 cc. per animal per day

In spite of the apparent health of these animals, certain of their tissues, when fed to planarian worms, proved nutritionally poor even to the point of producing severe disease. These tissues were liver, heart, and kidney cortex of guinea pigs which had been receiving as

supplements either orange juice, fresh tomato juice, or canned tomato juice. However, when kale or fresh grass was substituted for orange juice or tomato juice in the diet of the guinea pigs, their tissues were no longer nutritionally poor but were able to support growth and to maintain health in planarian worms.

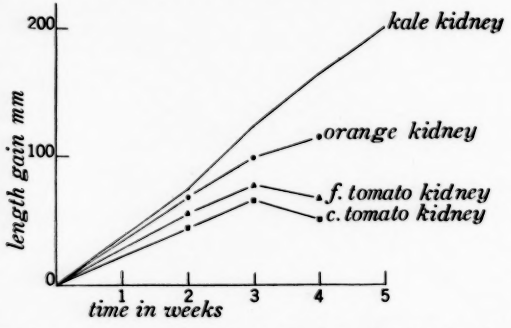

FIG. 7.—Each curve represents length gain in millimeters of thirty planarian worms fed the tissue diet specified on the curve.

Diets of guinea pigs furnishing tissues:

Basal diet

Ground barley	40 per cent
Skim milk powder, baked	26 per cent
Clarified butter	10 per cent
$CaCO_3$	3 per cent
NaCl	1 per cent

Basal-kale diet, yielding (kale) kidney:
Basal diet
Kale, 10 g. per animal per day

Basal-orange diet, yielding (orange) kidney:
Basal diet
Orange juice, 10 cc. per animal per day

Basal–fresh tomato diet, yielding (fresh tomato) kidney:
Basal diet
Fresh tomato juice, 10 cc. per animal per day

Basal–canned tomato diet, yielding (canned tomato) kidney:
Basal diet
Canned tomato juice, 10 cc. per animal per day

It might be suspected that some positive quality particularly damaging to planaria is imparted to the harmful guinea-pig tissues by the fruit juices were it not for the following observations. A group of guinea pigs was fed the basal diet regularly used in these experiments; and, as supplements, the guinea pigs were given both kale,

10 g. per animal per day, and also 10 cc. of canned tomato juice individually. After they had received the diet for a month, experimental groups of planaria were prepared and were fed liver, heart, and kidney from the guinea pigs over a period of 4 weeks. The worms were carefully examined throughout the experiment; and at all times and on all diets, they grew normally for the tissue concerned and remained entirely healthy. If the tomato juice had added an injurious element to the tissues of the guinea pigs, disease in the worms would have been widespread and severe at the end of the experiment.

To determine whether rats resembled guinea pigs in their response to kale supplement, two groups of rats were fed the same basal diet as guinea pigs and one group received, in addition, 3 g. of kale per animal per day. The other group received no supplement, as rats have no need for vitamin C. After the rats had been a month on the diet, their livers were fed to planarian worms, and the development of disease conditions in the worms was carefully watched. The rats often left a large part of their allotment of kale unconsumed, so that it is possible that they did not get the requisite quantity of supplement. Nevertheless, in the third week of planarian feeding practically all the worms fed (non-kale) liver showed unmistakable signs of the dietary disease, while none of the worms fed (kale) liver were affected. At the fourth week the disease had become severe throughout the worm group receiving (non-kale) liver, while it was just beginning to be manifest among the worms on the (kale) liver diet. By the sixth week, however, both sets of worms were hopelessly diseased.

Rat tissues therefore resemble guinea-pig tissues in their nutritional effects on planarian worms. Rat liver, otherwise injurious, can be rendered, to a certain extent, nutritionally suitable for planarian worms by using kale supplement; but the correction is not complete, owing quite possibly to the fact that the rats did not eat kale readily. It should be mentioned that rat spleen and lung are as harmless to planaria as are guinea-pig spleen and lung.

The evidence points to the existence of a factor—not one of the recognized vitamins—which must be present in the diet of guinea pigs if they are to produce liver, heart, and kidney cortex that are in

PLATE I

FIG. *A*.—Represents a group of thirty planarian worms after 5 weeks' feeding on kidney cortex derived from guinea pigs fed a scurvy-producing diet supplemented with fresh kale. All worms are normal.

FIG. *B*.—Similar group of thirty planarian worms after 5 weeks' feeding on kidney cortex derived from guinea pigs fed a scurvy-producing diet supplemented with canned tomato juice. All worms show the typical dietary disease.

PLATE I

good nutritional condition for planarian worms. As far as rats have been investigated, the same statement holds for them. This factor, which we shall call tentatively "factor *pl*," has been found in fresh green kale and grass but was found to be absent from dried alfalfa and from fresh and canned tomato juice and orange juice. There is apparently a very slight amount in orange juice but not enough to furnish adequate protection.

Why the various animal tissues possess such different amounts of factor *pl* can only be surmised. Either the spleen and lung might have stored such a large supply of factor *pl* that during the length of time of the experiment it was not depleted to a noticeable extent or these tissues may have a more tenacious hold on the factor than do kidney, liver, and heart.

Factor *pl* does not run parallel to growth-promoting power because spleen, which has high growth-promoting power, has high storage of *pl*, while liver, which has just as high growth-promoting power, has low storage of *pl*. On the other hand, lung, which has lower growth-promoting power than liver or spleen, has the best storage of *pl* of any tissue investigated; while heart, of almost equal growth-promoting power with lung, has poor storage of *pl*. The kidney, with only fair growth-promoting power, has the lowest storage of *pl* so far found. Even when kale was added to the basal diet, guinea pigs so fed provided kidneys not nutritionally perfect for planarian worms; and after 6 weeks' exposure to the kidney diet a small proportion of the worms showed slight dietary disease. It is, of course, possible that the presence of other chemical constituents of such a tissue as the kidney may modify the results.

Although factors promoting growth in planarian worms and factor *pl* do not appear to run parallel in animal tissues, nevertheless the groups of planaria in which dietary disease was ultimately to appear regularly exhibited lower growth than did their controls which were being supplied with tissue containing factor *pl*.

SUMMARY

1. Certain tissues of the guinea pig did or did not produce dietary disease in planarian worms, depending upon the diet of the guinea pigs furnishing the tissues.

2. If grass or kale were used as supplement to a basal diet nutritionally adequate except for the lack of vitamin C, guinea-pig tissues produced by this diet had correct nutritional properties for planarian worms.

3. If guinea pigs were fed as supplements orange juice or tomato juice in place of grass or kale, their livers, hearts, and kidneys produced severe dietary disease in planaria given a diet of these tissues. The guinea pigs themselves appeared entirely healthy.

4. Spleen and lung tissues proved to have good nutritional value for planaria when the guinea pigs producing the tissues were fed any of the supplements used: kale, fresh grass, orange juice, or tomato juice.

5. Evidence is assembled which points to the existence of a factor, not one of the recognized vitamins, which must be present in the diet of guinea pigs if they are to produce liver, heart, and kidney tissues which will support life in planarian worms.

6. There is some evidence that statement 5 is true for rat tissues also.

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THE EFFECTS OF TEMPERATURE ON THE DURATIONS OF THE DEVELOPMENTAL STAGES OF DROSOPHILA MELANOGASTER[1]

(Thirteen figures)

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HERETOFORE little accurate data have been presented to test the various theories offered to explain the effects of temperature on development. It is intended in the following report to provide such data, to test the relations between the various developmental stages, and to determine whether the existence of one part of a developmental stage at one temperature with the remainder of that stage at another temperature gives purely additive results on the basis of previously determined durations of the entire stage at each of these temperatures. With regard to the relations between stages, investigation is made into durations when both stages are spent at the same temperature, and when the second is spent at some temperature other than that of the first stage.

These data will be used to test the validity of the theories of Krogh, Van't Hoff, and Arrhenius as applied to development. It will be shown that none of these theories describes the facts presented; nor can other complete data on development be explained by these theories. An attempt will be made to demonstrate why these theories cannot be applied, that the facts are not such as can be described by

[1] For guidance and advice throughout this work I am indebted to Dr. C. R. Plunkett and also to my colleagues, Dr. A. L. Baron, Dr. O. S. Margolis, and Dr. G. P. Child.

Dr. Sewall Wright, who has been good enough to read this paper, has pointed out that "the fact that . . . . [the data] holds only for a particular inbred stock with a particular genetic constitution precludes the carrying-over of the results to other stocks. . . . ." This has been fully realized by the author and by other workers in this laboratory, with the result that this stock is being used as a basis for building up other stocks. Thus, various genes have been introduced by a method described by Baron (1935), so that the effects of single gene differences between stocks otherwise homogeneous can be examined.

them. Finally, a developmental scheme will be presented from which deductions concerning temperature effects can be made. A comparison will be made between these deductions and the data to see how closely they correspond.

MATERIALS AND METHODS

In nearly all previous data various possible sources of error were present. For example, in some cases no attention was paid to homogeneity of the material, or, in other cases, to uniformity or quality of culture conditions. In the present experiments all known or suspected sources of error were investigated to eliminate their entering into the results as uncontrolled variables. Wherever possible, environmental and genetic conditions were held constant, with the exception of the variable being investigated.

To check sources of error and to attain uniformity, it was necessary to inquire into (*a*) the uniformity of operation of apparatus and materials, (*b*) methods of handling, (*c*) genetic homogeneity, and (*d*) other, environmental factors difficult to classify. Despite all attempts to identify, or at least control, every possible source of error, there still remained (*e*) some residual uncontrolled variability. Each of these points will be discussed separately.

APPARATUS AND MATERIALS USED—UNIFORMITY OF OPERATION

The incubators were devised by Dr. Plunkett for just such experimental work as this. They are so similar to Bridges' (1932) "two-shelf incubators" that his description can be used. The incubators differed from his in a few minor respects; the relative advantages or disadvantages of these differences need not be discussed, since, as will be shown, temperature variations at any one point are probably too small to be important.

The regulator is also similar to the one employed by Bridges except that alcohol (95 per cent) is employed in place of toluene. This has the advantage of not forming an emulsion with the mercury, an emulsion which clings to the side of the capillary, causing a short at this point after some use; and at the same time alcohol behaves as sensitively to temperature changes as does toluene.

Where temperatures below that of the room was desired, the ther-

mostats were kept in a constant-temperature cold room maintained at about 10° C.

The temperature in these incubators was found to show two types of variability: variability from time to time at the same point and variability from point to point.

Variability in temperature at any one point is of the order of ±0.05° C. and is largely due to the low heat capacity of air. This source of variation was reduced to a negligible minimum by using small heaters which maintained the temperature without being on more than about half the time and by not leaving the incubators in too cold a room. By careful adjustment of heater to incubator and incubator to external temperature, and by stacking incubators in tiers of closely related temperatures, it was possible in some cases to reduce these fluctuations to ±0.01° C. (as, e.g., in the 14°–20° thermostats in the 10° cold room).

A second important cause of fluctuations in temperature at any one point was found to be changes in number and in disposition of vials and bottles in the incubator. These cause alterations in air currents and hence in rate of cooling. By keeping these changes at a minimum during any one experiment, this source of error was held to no more than 0.1° as a maximum, with nearly all experiments showing only 0.05° variation or less.

So far as the rhythmic variations resulting from the low heat capacity of the air are concerned, there is little effect on the temperature of the food medium. Using a thermocouple to measure the temperature of the air in the vial, of the mass of food, and of the surface of the food, as compared with the temperature in the air of the incubator (as measured with a thermometer), it was found that the temperature within the vial remained constant. The ordinary short-time fluctuations have, therefore, no discernible effect on the conditions under which the flies live.

Concerning the differences in temperature within the vial itself, it was found that the air of the vial and the mass of the food have practically the same temperature, but that the surface of the food may be as much as 0.02° lower than the mass. This is probably due to the small amount of evaporation which is constantly taking place from the surface to the nearly saturated air of the vial. The only

serious variation occurs when a small lump of moist material is left sticking to the thermocouple when it is removed from the body of the food to the air of the vial (but the vial not opened). This may be considered as equivalent to a larva crawling on the glass sides of the vial. The temperature of this mass falls as low as 0.3° below that of the vial. This error would be serious were it not true that the larva does not leave the food until just a few hours before pupation, and even then is in contact with the glass walls of the vial or bottle, from which it may absorb sufficient heat so that its temperature does not fall so low. Nevertheless, this remains one possible error.

Differences in temperature from place to place in the incubator are more serious and are affected by a greater variety of factors. Thus, the difference between lower and upper shelf may be as great as 0.5° C. Most of this difference seems to be caused by cooling (or heating) at the ends of the incubator. The simple expedient of placing a thermometer at the center of each shelf and of clustering the bottles and vials around the bulb of the thermometer eliminates this source of error, since, from point to point at any given area near the center of a shelf, there is little temperature difference.

Finally, as a check on the temperature and to determine the average for each experiment, the thermometer was read at least once each day.

For each experiment the bottles and vials were warmed or cooled to the desired temperature by leaving them in the incubators for some time before they were used. Where necessary, as when changing from one temperature to another, the temperature change was made by placing the containers in jars of water 1° above or below the desired temperature, as required, for 15 minutes. This has been found to effect more than 90 per cent of the desired change. The flies were then placed in the incubators.

The thermometers employed were calibrated in 0.1° intervals; and by means of a telescope reader, temperature could be estimated to ±0.01°. This apparent accuracy is valueless, however, unless the thermometers are standardized against a Bureau of Standards standardized instrument; and further, they must be restandardized at least once a year during the first few years of their life because of changes resulting from shrinkage of the glass bulb. These changes,

unless corrected, introduce errors of as much as 0.1° in many cases. In all the work in this laboratory the thermometers were so standardized.

The substratum used in these experiments was the usual mashed banana added to an equal amount of a hot (85°–90° C.) 4 per cent solution of agar. About 14 cc. were used in the 1×4-inch vials; 65 cc. in the half-pint milk bottles; and the containers slanted, as this gives a larger surface on which yeast may grow and the larvae crawl about than in unslanted bottles. Moreover, slants need not be plugged to prevent gas accumulation, and do not fall loose as readily as the usual flat food mass. On cooling, one drop of a thick yeast suspension is added.

A. L. Baron (1930) has already shown that this medium gives far more consistent results than corn meal–molasses, Pearl's S-101, or Pasteur's solution (to each of which live yeast suspension had been added). Experimentation with sterilized yeast-agar suspension showed such a medium to be more suitable for molds than flies.

The cotton stoppers were made loose enough to allow the freest possible ventilation with minimum drying of contents. It is doubtful if, at the most, the medium lost more than 15 per cent of its water, as estimated by appearance, though this was never tested by weighing.

Containers and stoppers were heat sterilized before being used.

METHODS OF HANDLING: (1) TIMING OF STAGES AND (2) CROWDING

In several respects the methods of handling, particularly timing of egg, larval, and pupal stages, are an improvement over those methods previously employed. Thus, eggs, pupae, and imagoes were counted in batches rather than as individuals—a method lending itself to great facility and hence increased speed and accuracy in timing. This is made clear from the following description:

1. In all work on the egg period, eggs were collected in 20- or 30-minute batches; and hatched eggs, recognized by their collapsed, wrinkled appearance, were counted every 20 or 15 minutes during eclosion. In this way the maximum error per egg introduced in timing was ±20 or ±22.5 minutes, respectively, with an average error

of 0 minutes. In a few experiments below 20° C. counts on hatching eggs were made every 30 or 40 minutes. This gives a maximum possible error for any egg of ±30 or ±35 minutes, with an egg-laying period of 30 minutes. In one case at 16° hatching eggs were counted every hour (laying period, 30 minutes; maximum possible error for any egg, therefore, ±45 minutes). As a result of such timing methods, the distribution of eggs hatching fell into six to nine groups at high temperatures (28°–30°) but were more than ten at lower (and still higher) temperatures. Since the distribution in time of eclosion is nearly symmetrical, very little error is introduced in the mean; but experimental σ, and hence the standard error, is too large by the amount of Sheppard's correction. This correction was not applied, since nothing was to be gained by a refinement of this type.

For experiments on the egg-larval period, the duration of egg-laying was invariably 1 hour. (It was found more convenient to measure the duration of the egg plus larval period ["egg-larval period"] than to determine the larval period as such. From this, all values for larval period were found by subtracting the duration of the egg period at the same temperature.) For temperatures above 20° the pupae were collected each hour; hence maximum possible error for any one larva is ±1 hour. At 20° and below to 16° the pupae were collected within 2 hours after pupation, at 15° every 6 hours; hence maximum possible individual errors were ±1.5 and ±3.5 hours, respectively. As in the egg, the distributions of lengths of larval periods were nearly symmetrical (except at temperature extremes), so that the errors tend to cancel each other.

In the pupal period counts were made of emerging flies every 3 hours at 15°; every 2 hours up to, and including, 20°; and above this temperature at every hour. These counts gave maximum possible individual pupal errors of ±4.5, ±2, and ±1 hour, respectively. The conclusions as to effect of grouping on mean and standard deviation are the same as for the egg and egg-larval periods.

Huettner (1923) has demonstrated that a female *Drosophila* may retain a fertilized egg for some time before it is laid. Hence, discarding the first egg laid by a female, all eggs laid in a given succeeding short period have been fertilized during this interval. It is not convenient, however, to collect eggs from individual flies. Instead, in

these experiments a number of pairs of flies were allowed to lay eggs on a suitable medium. The first batch of eggs was not used; but all subsequent batches were, and the time of fertilization taken as the middle of the interval of deposition. This transfer of flies from vial to vial (or bottle to bottle) could be continued for several hours, with the average female depositing from 4 to 8 eggs per hour even when transferred to fresh food every 20 or 30 minutes.

TABLE I

TWO EXAMPLES CHOSEN AT RANDOM TO SHOW DISTRIBUTION IN TIME OF HATCHING EGGS

EXPERIMENT 42a—25°

Duration of Egg Period (Hours)	No. of Eggs Hatching Each Interval	Duration of Egg Period (Hours)	No. of Eggs Hatching Each Interval
20.42	6	21.75	19
20.75	58	22.08	6
21.08	81	22.42	0
21.42	47		

EXPERIMENT 33—19°

Duration of Egg Period (Hours)	No. of Eggs Hatching Each Interval	Duration of Egg Period (Hours)	No of Eggs Hatching Each Interval
Before 35.75	1	38.5	10
36.0	1	39.0	2
36.5	2	39.5	1
37.0	11	40.0	3
37.5	30	40.5	0
38.0	33		

Using a number of females per vial or bottle in this manner does not eliminate the possibility of egg retention by some of the flies. However, the sharpness of distribution in time of hatching (see for egg period, Table VIII), added to the fact that very few eggs hatched much before the mass of eggs, is evidence that few eggs were long retained. Two examples chosen at random from the mass of data show this conclusion to be justified (Table I).

In order to secure large egg yields and facility in observation of

eggs upon the substratum, it was found useful to allow the females to lay eggs upon a medium consisting of 15 per cent molasses, 2 per cent agar, and the remainder, water. So far as yield goes, this medium is not better than ripe bananas; but the visibility of hatched and unhatched eggs on slants of this material is excellent. Yeast need not be added.

The fact that flies tend to retain their eggs when left upon an "old" (used, sour) food medium was utilized where large numbers of

TABLE II

DURATION OF EGG-LARVAL PERIOD (IN HOURS) FROM EGGS LAID IN SUCCESSIVE HOURS

Temp. °C	First Hour	Second Hour	Third Hour	Fourth Hour	Fifth Hour
15.24	393±1.3	395±1.5	399±1.4	400±1.7†	405±3.6
29.98	104.4±0.43	106.6±0.33	107.3±0.61*	107.8±1.1	103.2±0.66
22.00	141.6±0.21	141.7±0.18	.....	.....	.....
	149.9±0.17	149.9±0.16	.....	.....	.....
27.77	94.4±0.13	94.8±0.14	.....	.....	.....
	93.6±0.12	94.3±0.16	.....	.....	.....

Temp. °C	Sixth Hour	Seventh Hour	Eighth Hour	Ninth Hour	Tenth Hour
15.24	395±1.9	399±3.1	400±2.0†	385±4.3	390±3.6
29.98	101.5±0.89	103.6±0.80	.....	.....	.....

* Six-hour interval between third and fourth hours when no eggs were collected.

† Five-hour interval follows points marked.

NOTE.—These are unusually good agreements, considering the variability commonly found from experiment to experiment; and, furthermore, there is no trend.

eggs were to be collected over several hours. This was most easily accomplished by leaving the flies in their mating vials till they were used for experiments. By this time the food was well "chewed up" and worked over by the F_1 larvae. There was no indication that eggs laid by these flies were different from eggs which had not been long retained. Thus, in several experiments eggs were used which were laid over a number of consecutive hours (Table II).

The previous cumbersome method of timing pupae by placing each one in a separate vial was considerably simplified in these experiments. Each hour's batch of pupae coming from eggs laid at

the same time were placed upon an agar (2 per cent agar in water) slant in the same vial. All these pupae have had the same egg-larval period. The distributions in time and sex of the emerging imagoes were then determined at regular intervals. In this way the number of vials handled was little more than the number of hours during which pupation occurred, rather than being equal to the total number of pupae collected. Furthermore, facility of manipulation and of distribution in the incubator was greatly increased, and change in temperature resulting from removal for counting greatly diminished by this method.

2. For most of the experiments the larval period was run in the 1×4-inch vials. To check the effect of crowding on the duration of this period the averages for vials having different numbers of larvae were compared with each other. When the eggs were all laid during a 1-hour interval, there was no statistically significant regression coefficient between the durations whether 7 larvae were present or 57 (see Table III). Nevertheless, in each experiment the attempt was made to keep the number of eggs per vial at between 15 and 30.

TABLE III

EFFECT OF CROWDING ON DURATION OF EGG-LARVAL PERIOD

Regression coefficient = +0.09±0.09

No. of Larvae per Vial	No. of Vials	Mean Egg-Larval Period±s.e. (Hours)	No. of Larvae per Vial	No. of Vials	Mean Egg-Larval Period±s.e. (Hours)
7–10	3	100.3±0.69	31–35	5	104.9±0.31
11–15	2	105.0±0.93	36	1	104.3±0.62
16–20	6	105.2±0.34	43	2	105.1±0.86
21–25	4	101.3±0.45	48	1	107.0±0.42
26–30	5	104.2±0.33	57	1	105.9±0.39

In later experiments the far more convenient method of using bottles containing up to about 250 eggs was used. Here it was necessary to add crumbled yeast cake from time to time as the growing larvae consumed it. Dr. G. P. Child (1935) has found this method to give very uniform results with scute. In the present experiments no simultaneous check between such conditions and those in vials was made; but where experiments had been run by both methods

at the same, or nearly the same, temperature, it was found that the means and coefficients of variation were as close as is ordinarily obtained from experiment to experiment. In the tables those experiments carried out in bottles are marked ([B]).

GENETIC FACTORS—HOMOZYGOSITY

The flies used in these experiments were inbred in pair matings for more than 3 years. Such a degree of inbreeding (about seventy-five generations) may be expected to give as homogeneous a stock as could be desired. Several facts indicate that a high degree of homozygosity has been attained. Thus, σ declined from $\pm 1.42 \pm 0.07$ as a minimum value after six generations of inbreeding to ± 1.20-

TABLE IV

PARENT-OFFSPRING CORRELATION COEFFICIENT
Egg-larval periods, P_1 at 25.56°, F_1 at 25.14°

P_1		F_1	P=O
Males (Hours)	Females (Hours)	Both Sexes Combined	Correlation
107.1 ± 0.17	107.8 ± 0.21	122.0 ± 0.48	Males=0.00
108.0 ± 0.23	108.2 ± 0.22	123.5 ± 0.38	
109.7 ± 0.38	109.4 ± 0.38	122.0 ± 0.21	Females=0.00
110.4 ± 0.24	111.5 ± 0.25	121.4 ± 0.22	

± 0.06 as an average value in later experiments (pupal period, 25°). Again, in another experiment in which this stock was compared with another, provided by A. L. Baron (1935), which had been "made homozygous" by a method described by him, the inbred stock gave for the egg-larval period $\sigma_{\sigma^{\prime}} = \pm 2.3 \pm 0.2$ and $\sigma_{\varphi} = \pm 2.8 \pm 0.2$ when the "homozygous" stock gave $\sigma_{\sigma^{\prime}} = \pm 4.1 \pm 0.3$ and $\sigma_{\varphi} = \pm 3.2 \pm 0.2$. In the same experiment it was observed (qualitatively) that the first and last hatched of the inbred flies were equally viable and fertile; while in the "homozygous" stock, of the last 10 per cent of the flies hatched, about one-half died in a few days and the remainder laid very few eggs.

Perhaps the most important evidence of homozygosity was the parent-offspring correlation shown in Table IV.

The stock may be considered, therefore, sufficiently homogeneous and homozygous.

OTHER, ENVIRONMENTAL FACTORS

Several other factors were suspected of modifying the rate of development. These are (1) age of parents, (2) conditions under which parents are aged, (3) conditions of development of parents, and (4) a diurnal rhythm in emergence.

1. Li (1927) concluded from his data that the duration of the egg-larval period was negatively correlated with the age of the parents. To check this point, eggs were collected from a number of pairs of flies when they were 3 days old and again when they were 7 days old. These gave egg-larval periods of 105.6 ± 0.74 and 106.5 ± 0.24 hours, respectively; the difference is not significant. To avoid, nev-

TABLE V

DURATION OF EGG PERIOD IN HOURS

Age of Flies (Days)	Flies from Fresh Food	Flies on Original Food
5–7	20.39 ± 0.064	
8–10	20.41 ± 0.037	20.94 ± 0.042
15–17	21.15 ± 0.050	22.31 ± 0.13

ertheless, any possible error on this score, flies were used in the following experiments which were about 6 days old; but in some cases they were as young as 4 or as old as 10 days.

2. The possibility of age affecting duration does exist, however, since, as the flies remain in the mating vials, the food gets older and more acid. Such conditions act adversely and rapidly reduce the viability and fecundity of the flies, so that they lay few eggs and soon die. Whether this affects the duration of development of (at least) the egg period was tested by timing the eggs from two groups of flies, one of which was several times changed to fresh food, the other remaining on the original food in the mating vials. The results are shown in Table V. Transfers to fresh food were made after the first and second experiments.

It is obvious from such results that the duration of embryonic development is correlated with the "age" of the parents probably

only when the parents subsist upon poor food. The increase in the second column between the last two figures is due, probably, to the same factor, since only two transfers were made to fresh food. The use of flies less than 9 days old in nearly all of these experiments obviated this source of error.

3. It was observed by several workers in this laboratory that parents from crowded bottles did not lay as well as those from bottles having sufficient food for the P_1 larvae (i.e., having an excess of yeast always visible). There were also indications that the offspring of this first class of parents did not give uniform results from experiment to experiment. In view of the fact that starved flies lay smaller eggs than normal flies do, such results are not unlikely. Though no detailed examination was made of this possible source of error, care was taken to grow the parents in uncrowded bottles (100–200 eggs) with yeast added from time to time; and these bottles were always kept in the incubator at 25°.

4. As a result of Bliss's (1926) observations it is generally believed that the length of the pupal period is in some way related to the time of day of emergence, or, as Bliss puts it, "Although equal numbers of larvae formed puparia every hour throughout the day, the adults from these puparia did not similarly emerge in equal numbers." This point was several times checked by me as a preliminary to the following experiments, and results like those of Bliss were obtained. Bonnier (1926) records similar data in the form of a larval-pupal correlation coefficient. Table VI shows typical results.

In later experiments, however, even duplicating as closely as possible those conditions of experimentation inherent in the earlier ones, it was found impossible to repeat these results (see Table XII). For if, in the experiments presented in Table XII, a diurnal rhythm was present, the first-formed pupae would have shown a different duration of pupal life from the later-formed pupae; hence a correlation coefficient would be consistently present between the larval and pupal periods. Such a correlation is not consistently found. Dr. Darby, using this stock, has arrived at the same conclusions. Both the factors of crowding and heterozygosity (factors inherent in the above mentioned experiments) were checked and found not to be the cause of this rhythm. It seems, therefore, that in the later ex-

periments the method of experimentation is, in some unaccounted way, different from that of Bliss or of my earlier work. This absence

TABLE VI

EFFECT OF TIME OF DAY OF PUPATION ON DURATION OF PUPAL PERIOD

Time of Day of Puparium Formation (Hours)	Mean Pupal Life			
	Males		Females	
	Hours ± s.e.	No.	Hours ± s.e.	No.
0–1	.....	.....	.....	.....
1–2	101.6±0.89	3	92 ±0	2
2–3	103.1±0.2	37	97.2±0.7*	29
3–4	103.1±0.2	33	98.9±0.4	39
4–5	102.3±0.2	27	98.7±0.4	26
5–6	100.3±0.3	3	98.0±0	3
6–7	100.4±0.6	5	97.5±0.5	2
7–8	.....	.....	.....	.....
8–9	99.2±0.3	6	96.6±0.2	8
9–10	100	1	97	1
10–11	99.0±0.2	5	96 ±0	2
11–12	97.8±0.4	10	95.3±0.2	9
12–13	97.2±0.3	15	94.5±0.2	19
13–14	97.5±0.2	30	94.6±0.3	21
14–15	96.6±0.2	44	93.3±0.2	40
15–16	97.2±0.3	9	93.9±0.2	15
16–17	96.4±0.2	14	93.4±0.3	10
17–18	94.9±0.2	59	92.3±0.1	56
18–19	95.6±0.3	26	91.5±0.2	20
19–20	96.8±0.9	5	91.5±0.9	4
20–21	95.5	1	92.5±0.5	2
21–22	97.8±0.5†	59	91.4±0.2	36
22–23	.....	.....	93.5±0	2
23–24	.....	.....	92.2±0.3	5

* The individuals included in this group fell into two distinct subgroups, one subgroup of 6 individuals averaging 90.8±0.5 hours, and the other, of 23 individuals, averaging 98.9±0.4 hours.

† The individuals included in this group fell into two distinct subgroups, one subgroup of 38 individuals averaging 94.5±0.2 hours, and the other, of 21 individuals, averaging 102.2±0.5 hours.

NOTE.—Time of puparium formation is used, rather than time of emergence, since the latter is bound to give a positive correlation with small numbers whether the relation is real or not. This is due to the fact that later-emerging flies must be older than the earlier-emerging ones.

of diurnal rhythm in the experiments presented, however, reduces the experimental and theoretical difficulties.

RESIDUAL VARIABILITY

Despite all precautions, variability could not be completely eliminated, or even accounted for. This is particularly true for the egg-larval period, though the other stages show similar, but smaller, variations. Thus one series of experiments at 25.14° gave (arranged in increasing order) the values shown in Table VII.

TABLE VII

RESIDUAL VARIABILITY—A SERIES OF EXPERIMENTS AT 25.14° C.

Exp. No.	Egg-Larval Period				Pupal Period	
	Males		Females		Males	Females
	Hours ± s.e.	$\frac{\sigma}{\text{Mean}}$	Hours ± s.e.	$\frac{\sigma}{\text{Mean}}$	Hours ± s.e.	Hours ± s.e.
1	107.7 ±0.13	0.014	108.5 ±0.21	0.014	96.7 ±0.11	94.0 ±0.08
2	111.2 ±0.13	0.019	110.9 ±0.16	0.024	97.3 ±0.08	93.3 ±0.12
3	113.4 ±0.26	0.022	114.5 ±0.29	0.026	95.6 ±0.12	93.0 ±0.12
4	113.5 ±0.57	0.050	113.5 ±0.44	0.043	97.0 ±0.13	94.9 ±0.09
5	116.6 ±0.42	0.041	117.4 ±0.38	0.035	96.1 ±0.11	93.4 ±0.11
6	117.4 ±0.33	0.034	118.9 ±0.30	0.033	96.2 ±0.10	93.4 ±0.10
7	118.7 ±0.35	0.032	119.4 ±0.34	0.030	95.8 ±0.10	93.2 ±0.13

In the foregoing experiments the conditions were as follows: Experiments 1 and 2 were carried out with the same bunch of ripe bananas; experiments 5, 6, and 7 with another lot; experiments 3 and 4 on two lots of green bananas. The agar solution was at 95° C. when bananas were added in experiments 3, 5, and 6 and at 85° C. in experiment 7.

Although there can be little doubt that the differences are due to some uncontrolled variables in the medium, it is obvious that these are not the degree of ripeness of the bananas used or the temperature of the agar solution when the bananas are added; nor is the yeast likely to be an important cause, since the same yeast was used in Experiments 1 and 4 and another cake for Experiments 3, 5, 6, and 7.

The fact that such variability is found almost entirely in the

larval, and only to a smaller extent in the egg or pupal periods, is in itself evidence that the cause of this variability is in the substratum on which the larvae live. This will probably be eliminated by the discovery of a medium the components of which will remain constant from experiment to experiment and during each experiment. The ideal would be a synthetic medium, preferably of simple substances, but in any case with the concentration of materials added well under control. As Baron (1930) has found, however, such media have so far proved to give more, rather than less, variable results.

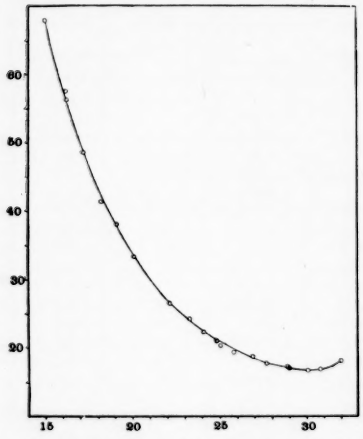

FIG. 1.—Effect of temperature on the duration of the embryonic period. Abscissa, temperature; ordinate, duration in hours.

THE DATA

The duration of the embryonic period is given in Table VIII; the column headed "Duration" represents the experimental facts. Since the eggs were always laid at 25°, a correction should be made to the observed time to allow for the average period spent at that temperature. This correction is made on the assumption that equal fractions of time at different temperatures represent correspondingly equal amounts of development. This assumption is not altogether justified, but gives a "corrected" value for the egg period much closer to that to be expected if each egg were placed at the experimental temperature immediately upon being laid. This corrected value is presented in the last column of the table and is shown graphically in Figure 1. All curves are drawn by eye as the nearest fit to the points.

Some of the points deviate significantly, as measured by their standard errors, from the smooth line drawn. These deviations cannot be accounted for; but since they fall to either side of the line for eggs laid at the same time by the same parents (cf. points with experiment number) and since they nowhere deviate by more than

1.4 per cent, any conclusions which may be drawn to within this limit may be valid.

A single attempt was made to determine whether any sex dimorphism in time of development could be demonstrated. The eggs, kept at 28° until about half had hatched, were rapidly cooled and

TABLE VIII

EMBRYONIC PERIOD

Temp. °C.	Exp. No.	Durations (Hours)	No. of Individuals	σ	Eggs Laid at—		Corrected Egg Period (Hours)
					Temp. °C.	During (Hours)	
14.95.....	25	67.5 ±0.33	54	2.41	25.1	0.33	67.9
16.16.....	44	57.1 ±0.12	182	2.28	25.0	0.50	57.6
16.19.....	26	56.0 ±0.12	153	1.46	25.1	0.33	56.3
17.15.....	28	48.4 ±0.12	129	1.40	25.1	0.50	48.7
18.20.....	25	41.2 ±0.16	64	1.30	25.1	0.33	41.4
19.08.....	33	37.80±0.059	94	0.57	25.1	0.50	38.01
20.07.....	28	33.33±0.080	82	0.73	25.1	0.33	33.44
22.14.....	25	26.50±0.083	57	0.63	25.1	0.33	26.54
23.27.....	28	24.24±0.038	135	0.44	25.1	0.50	24.28
24.09.....	33	22.44±0.029	188	0.40	25.1	0.50	22.46
24.81.....	42	21.13±0.017	217	0.36	25.0	0.50	21.14
24.84.....	40	21.05±0.027	141	0.46	25.0	0.50	21.05
25.06.....	27	20.39±0.064	37	0.38	25.1	0.50	20.39
25.06.....	27	20.41±0.037	84	0.34	25.1	0.50	20.41
25.80.....	26	19.45±0.026	196	0.36	25.1	0.33	19.44
26.92.....	33	18.77±0.029	104	0.30	25.1	0.50	18.75
27.68.....	26	17.79±0.041	148	0.49	25.1	0.33	17.76
28.89.....	29	17.38±0.043	83	0.39	25.1	0.25	17.36
28.96.....	40	17.26±0.031	95	0.43	25.0	0.50	17.23
29.00.....	44	17.18±0.023	232	0.50	25.0	0.50	17.14
30.05.....	26	16.81±0.032	148	0.39	25.1	0.33	16.77
30.80.....	26	16.97±0.028	195	0.39	25.1	0.33	16.94
32.0.....	33	18.20±0.29	58	2.23	25.1	0.50	18.17

the unhatched eggs removed. Both hatched and separated eggs were allowed to complete their development. The ratios found were:

	Males	Females
Early-hatched eggs.....	0.41±0.075	0.59±0.075
Late-hatched eggs.....	0.38±0.11	0.62±0.11

Thus, for the small numbers used in this experiment there is no significant difference between the sexes.

TABLE IX

EGG-LARVAL PERIOD

TEMP. °C.	EGG-LARVAL PERIOD (FOUND)						EGG-LARVAL PERIOD (CORRECTED)		EGG PERIOD FROM CURVE	LARVAL PERIOD		DIFF. F.—M.
	Male			Female						Male (Hours)	Female (Hours)	
	Hours ± s.e.	No.	σ	Hours ± s.e.	No.	σ	Male	Female				
14.86	421.4 ± 1.68	97	16.3	423.8 ± 1.56	114	16.4	422.8	425.2	69.0	353.8 ± 1.6	356.2 ± 1.5	+ 2.4
15.24	394.6 ± 0.89	227	13.4	399.1 ± 0.82	227	12.2	395.8	400.4	65.2	330.6 ± 0.88	335.2 ± 0.79	+ 4.6
16.06[B]	328.6 ± 0.91	148	11.0	342.2 ± 0.62	187	8.3	329.6	343.3	57.8	261.8 ± 0.90	285.5 ± 0 60	+13.72
18.04	250.1 ± 0.70	99	7.0	362.7 ± 0.76	76	6.6	250.8	264.5	42.5	208.3 ± 0.69	222.0 ± 0.74	+13.7
18.05[B]	241.3 ± 0.32	423	6.8	259.1 ± 0.32	407	6.6	241.9	259.8	42.4	199.5 ± 0.10	217.4 ± 0.10	+17.9
18.05[B]	243.2 ± 0.53	178	7.1	262.4 ± 0.50	178	6.7	243.9	263.1	42.4	201.5 ± 0.52	220.7 ± 0.20	+19.2
18.21	242.2 ± 0.73	81	6.6	258.7 ± 0.60	84	5.5	242.8	259.3	41.5	201.3 ± 0.72	217.8 ± 0.58	+16.5
19.32[B]	207.7 ± 0.36	122	3.9	222.2 ± 0.26	127	3.0	208.2	222.8	36.4	171.8 ± 0.11	186.4 ± 0.26	+14.6
19.97	188.0 ± 0.71	125	8.0	202.6 ± 0.82	138	9.7	188.3	203.0	33.8	154.5 ± 0.71	169.2 ± 0.82	+14.7
22.00	141.7 ± 0.18	128	2.0	149.9 ± 0.16	129	1.8	141.8	150.1	26.9	114.9 ± 0.17	123.2 ± 0.15	+ 8.3
22.00	141.6 ± 0.21	195	3.0	149.9 ± 0.17	183	2.3	141.7	150.1	26.9	114.8 ± 0.21	123.2 ± 0.16	+ 8.4
22.21	146.1 ± 0.31	139	3.6	153.1 ± 0.21	152	2.6	146.2	153.3	26.4	119.8 ± 0.30	126.9 ± 0.20	+ 7.1
22.99[B]	130.5 ± 0.19	220	2.9	139.8 ± 0.22	216	3.2	130.6	139.9	24.7	105.9 ± 0.19	115.2 ± 0.22	+ 9.3
24.17[B]	118.5 ± 0.29	140	3.4	120.7 ± 0.21	185	2.9	118.5	120.7	22.3	96.2 ± 0.29	98.4 ± 0.21	+ 2.2
24.93[B]	113.3 ± 0.14	242	2.2	113.6 ± 0.16	229	2.4	113.3	113.6	20.9	92.4 ± 0.14	92.7 ± 0.16	+ 0.3
24.93[B]	112.2 ± 0.13	341	2.4	113.4 ± 0.14	337	2.5	112.2	113.4	20.9	91.3 ± 0.13	92.5 ± 0.13	+ 1.2
25.14*	113.7 ± 0.15	1,004	(4.6)	114.2 ± 0.16	1,039	(5.2)	113.7	114.2	20.6	93.1 ± 0.14	93.6 ± 0.16	+ 0.5
25.56	108.3 ± 0.14	357	2.6	108.3 ± 0.15	359	2.8	108.3	108.3	20.0	88.3 ± 0.14	88.3 ± 0.15	0.0
25.99[B]	105.5 ± 0.14	344	2.6	105.8 ± 0.15	298	2.8	105.5	105.8	19.4	86.1 ± 0.14	86.4 ± 0.16	+ 0.3
26.89[B]	100.1 ± 0.15	185	2.1	99.9 ± 0.18	203	2.6	100.0	99.8	18.5	81.5 ± 0.12	81.3 ± 0.18	− 0.2
27.77	94.8 ± 0.14	128	1.5	94.3 ± 0.16	123	1.8	94.7	94.2	17.8	76.9 ± 0.13	76.4 ± 0.16	− 0.5
27.77	94.4 ± 0.13	192	1.3	93.5 ± 0.12	207	1.7	94.3	93.4	17.8	76.5 ± 0.09	75.6 ± 0.12	− 0.9
28.07	103.0 ± 0.34	74	2.9	102.9 ± 0.31	63	2.4	103.0	102.9	17.7	85.3 ± 0.32	85.3 ± 0.30	− 0.1
28.99	98.4 ± 0.25	98	2.4	98.4 ± 0.28	96	2.7	98.3	98.3	17.2	81.1 ± 0.24	81.1 ± 0.27	0.0
29.47	99.2 ± 0.30	82	2.7	98.4 ± 0.32	88	3.0	99.1	98.3	17.0	82.1 ± 0.30	81.3 ± 0.32	− 0.8
29.98	105.1 ± 0.25	226	3.7	105.1 ± 0.31	242	4.7	105.1	105.1	16.8	88.3 ± 0.24	88.3 ± 0.30	0.0
31.04	121.4 ± 0.56	157	7.1	118.5 ± 0.55	188	7.5	121.5	118.5	17.1	104.4 ± 0.57	101.4 ± 0.55	− 3.0

[B] Experiments in one-half pint milk bottles.

* Average of experiments presented in Table VII.

The egg and larval periods were determined as a unit, and the larval period obtained from this by subtraction of the egg period at the corresponding temperature. The results are presented in Table IX. As in the case of the embryonic period, the egg-larval period is corrected for the average time spent at 25°, the temperature at which

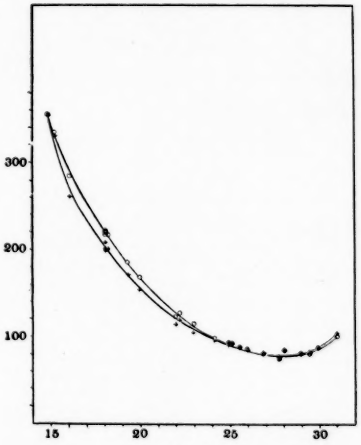

FIG. 2

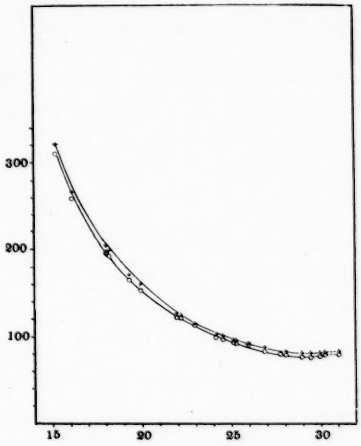

FIG 3

FIG. 2.—Effect of temperature on the duration of the larval period. Abscissa, temperature; ordinate, duration in hours. + = male values; ○ = female values.

FIG. 3.—Effect of temperature on the duration of the pupal period. Abscissa, temperature; ordinate, duration in hours. + = male values; ○ = female values.

the eggs were layed. From this the duration of the egg period, obtained from the corrected curve, is subtracted, thus giving the larval period alone.

The standard deviation for the larval period is derived from

$$\sigma^2_{\text{egg-larva}} = \sigma^2_{\text{egg}} + \sigma^2_{\text{larva}} ,$$

on the assumption that there is no correlation between egg and larval stages. Hence

$$\sigma^2_{\text{larva}} = \sigma^2_{\text{egg-larva}} - \sigma^2_{\text{egg}} .$$

The values of $\sigma_{egg\text{-}larva}$ and σ_{egg} are experimentally determined, the latter being taken as the value at points nearest the desired temperature.

The last column in Table IX shows the difference in duration between male and female larvae at the same temperature. These vary considerably with temperature and even change in sign at the upper end of the temperature range.

TABLE X

PUPAL PERIOD

Temp. °C.	Male			Female			Diff. M.−F.
	Hours ± s.e.	No.	σ	Hours ± s.e.	No.	σ	
15.24	320.5 ±0.45	228	6.72	309.5 ±0:42	227	6.30	+11.0
16.17	266.7 ±0.30	76	2.62	259.0 ±0.24	186	3.16	+7.7
18.01	204.4 ±0.28	97	2.78	195.3 ±0.26	76	2.26	+9.1
18.05	204.4 ±0.15	178	2.06	197.0 ±0.14	174	1.81	+7.4
18.21	199.2 ±0.22	83	2.01	192.2 ±0.29	84	2.67	+7.0
19.32	170.6 ±0.17	120	1.87	164.7 ±0.10	126	1.12	+5.9
19.97	160.1 ±0.21	125	2.40	152.2 ±0.33	138	3.86	+7.9
22.00	126.5 ±0.13	195	1.79	121.4 ±0.14	182	1.91	+5.1
22.21	124.04 ±0.089	138	1.05	120.70 ±0.082	152	1.01	+3.34
22.99	115.62 ±0.089	153	1.09	113.08 ±0.074	215	1.10	+2.58
24.17	102.4 ±0.14	140	1.64	98.65 ±0.074	185	1.02	+3.75
24.57	100.51 ±0.080	238	1.25	96.78 ±0.065	229	1.00	+3.73
25.14	96.51 ±0.044	967	1.37	93.55 ±0.042	1,018	1.35	+2.96
25.29	96.40 ±0.096	99	0.959	92.23 ±0.15	118	1.59	+4.17
25.99	92.24 ±0.060	342	1.12	90.20 ±0.058	298	1.01	+2.04
26.89	87.23 ±0.075	185	1.02	82.56 ±0.069	203	0.99	+4.67
27.77	.....	.....	.....	80.0 ±0.11	120	1.21	.....
28.07	83.2 ±0.16	72	1.38	78.6 ±0.13	64	1.07	+4.6
28.99	80.9 ±0.11	85	0.99	76.2 ±0.12	82	1.08	+4.7
29.47	80.6 ±0.12	77	1.05	75.8 ±0.11	70	0.92	+4.8
29.98	81.3 ±0.11	233	1.68	77.1 ±0.10	246	1.60	+4.2
30.24	82.0 ±0.13	141	1.50	78.5 ±0.12	161	1.50	+3.5
31.04	82.7 ±0.17	73	1.4	79.3	22	.....	+3.4

Data on the pupal period are given in Table X and presented graphically in Figure 3. Here no corrections had to be applied. The difference between males and females (last column) remains practically proportional to the duration of development at all temperatures.

The interstage ratios derived from the foregoing data are shown in Table XI. Although fairly constant, a trend is to be noted, par-

ticularly at the lowest and highest temperatures. This trend is least noticeable in the ratio of embryonic period to pupal period. The correlation coefficients (all those calculated) are given in Table XII. Of these eighteen values, only six show a possible interstage correlation. Of these, four (Exps. 4, 5, 8, 9) are only four times their standard

TABLE XI

RATIOS BETWEEN THE THREE DEVELOPMENTAL STAGES

Temp. °C.	Embryonic Period / Larval Period		Larval Period / Pupal Period		Embryonic Period / Pupal Period	
	Male	Female	Male	Female	Male	Female
14.86	0.195	0.194	.....	.....	.....	.....
15.24	0.197	0.195	1.03	1.08	0.203	0.211
16.06	0.221	0.202	0.982	1.10	0.217	0.223
18.04	0.204	0.190	1.02	1.14	0.208	0.221
18.05	0.213	0.195	0.986	1.12	0.210	0.215
18.21	0.205	0.191	1.01	1.13	0.208	0.216
19.32	0.212	0.195	1.01	1.13	0.214	0.221
19.97	0.219	0.200	0.965	1.11	0.211	0.222
22.00	0.234	0.218	.....	.....	.....	.....
22.00	0.234	0.218	0.907	1.01	0.212	0.222
22.21	0.220	0.208	0.966	1.05	0.212	0.219
22.99	0.233	0.214	0.917	1.02	0.214	0.218
24.17	0.232	0.227	0.939	0.997	0.218	0.226
24.57	.....	.....	.....	.....	0.213	0.222
24.93	0.228	0.226	.....	.....	.....	.....
25.14	0.221	0.220	0.965	1.00	0.213	0.220
25.56	0.227	0.227	.....	.....	.....	.....
25.99	0.226	0.225	0.934	0.958	0.211	0.215
26.89	0.227	0.228	0.934	0.985	0.211	0.223
27.77	0.233	0.235	.....	0.955	.....	0.223
28.99	0.211	0.211	1.00	1.06	0.213	0.226
29.47	0.207	0.209	1.02	1.07	0.211	0.225
29.98	0.190	0.190	1.09	1.15	0.207	0.219
31.05	0.164	0.168	1.26	1.28	0.207	0.216

NOTE.—Embryonic period values taken from Figure 1.

error, with two of one sign and two of the other; but the other two values (Exp. 6) are about ten times their standard error. The significance of these two values is not clear; especially since Exps. 2 and 5 were carried out at the same time and with eggs from the same parents on the same food. The distributions were not comparable to those of the previously found "diurnal rhythms."

Many other experiments were plotted in correlation diagrams;

none showed any obvious relation between the egg-larval and pupal stages.

When larvae are crowded, their development is prolonged. The pupae which they produce are visibly smaller than normal and take

TABLE XII

CORRELATION COEFFICIENT BETWEEN EGG-LARVAL AND PUPAL PERIODS

Exp. No.	Temp. °C.	No. of Males and Females	r±s.e.
1	15.24	227 f	−0.003±0.04
2	18.05	178 m, 178 f	m=+0.01±0.08, f=+0.01±0.08
3	18.21	81 m	+0.01±0.11
4	22.00	195 m, 183 f	m=−0.07±0.07, f=+0.29±0.07
5	22.99	220 m	−0.22±0.06
6	24.93	242 m, 229 f	m=−0.54±0.05, f=−0.46±0.05
7	25.14	280 m	−0.12±0.06
8	25.14	130 m	−0.29±0.07
9	25.14	98 m	+0.34±0.09
10	25.14	127 m	+0.16±0.09
11	25.14	159 m	+0.16±0.17
12	25.14	91 m	−0.05±0.10
13	25.14	119 m	+0.03±0.09
14	26.79	166 m	+0.19±0.10
15	27.77	123 f	−0.03±0.09

TABLE XII*A*

EFFECT OF CROWDING ON LARVAL-PUPAL CORRELATION COEFFICIENT (MALES PRESENTED)

	Uncrowded (Hours)	Crowded (Hours)
Egg-larval period	111.2 ±0.13	188.1 ±0.68
Pupal period	97.3 ±0.08	104.2 ±0.19
Correlation coefficient	−0.12±0.06	+0.69±0.05

longer to develop (Table XII*A*). Under such conditions a marked larval-pupal correlation appears.

The duration of the egg-larval period of the crowded flies was actually much longer, for only those larvae which pupated during the first 2 days after pupation started were collected; the remainder continued to pupate for more than a week.

The ratios, σ divided by the mean, are given in Table XIII. For

the larger part of the range, σ/mean remains nearly constant, but is considerably higher at the extremes of temperature.

Table XIV gives the duration of the pupal period when the egg-larval period is spent either at the same temperature or at some

TABLE XIII

VALUES OF σ/MEAN

Temp. °C.	Embryonic Period	Temp. °C.	Egg-Larval Period		Pupal Period	
			Male	Female	Male	Female
14.95	0.036	14.86	0.0387	0.0387	.....	.....
16.16	0.040	15.24	0.0339	0.0306	0.0210	0.0204
16.19	0.026	16.06	0.0334[B]	0.0242[B]	0.0098	0.0122
17.15	0.029	18.04	0.0281	0.0248	0.0136	0.0116
18.20	0.032	18.05	0.0280[B]	0.0255[B]	.....	.....
19.08	0.015	18.05	0.0291[B]	0.0256[B]	0.0101	0.0092
20.07	0.022	18.21	0.0273	0.0214	0.0101	0.0140
22.14	0.024	19.32	0.0188[B]	0.0135[B]	0.0109	0.0067
23.27	0.018	19.97	0.0425	0.0478	0.0150	0.0127
24.09	0.018	22.00	0.0142	0.0121	.....	.....
24.81	0.017	22.00	0.0209	0.0156	0.0141	0.0158
24.84	0.022	22.21	0.0247	0.0170	0.0084	0.0084
25.06	0.019	22.99	0.0218[B]	0.0226[B]	0.0095	0.0096
25.06	0.017	24.17	0.0287[B]	0.0236[B]	0.0161	0.0103
25.80	0.018	24.57	.....	.....	0.0125	0.0103
26.92	0.016	24.93	0.0196[B]	0.0209[B]	.....	.....
27.68	0.028	24.93	0.0210[B]	0.0223[B]	.....	.....
28.89	0.022	25.56	0.0244	0.0263	.....	.....
28.96	0.022	25.29	.....	.....	0.0095	0.0172
29.00	0.029	25.99	0.0244[B]	0.0263[B]	0.0121	0.0112
30.05	0.023	26.89	0.0209[B]	0.0261[B]	0.0117	0.0120
30.80	0.023	27.77	0.0164	0.0193	.....	0.0134
32.0	0.12	27.77	0.0136	0.0183	.....	.....
		28.07	0.0279	0.0236	0.0166	0.0136
		28.99	0.0247	0.0274	0.0122	0.0142
		29.47	0.0273	0.0305	0.0130	0.0121
		29.98	0.0353	0.0451	0.0207	0.0208
		30.24	.....	.....	0.0183	0.0191
		31.04	0.0583	0.0628	0.0169	.....

[B] Flies grown in one-half pint milk bottles.

other temperature. In the transfer from 22° to 28°, or in the converse transfer, the temperature at which the egg-larval period developed does not affect the duration of the pupal period. In the transfer from 25° to 18° and its converse, the pupal period is longer when the egg-larval period is spent at the lower temperature.

In Tables XV and XVI are presented the data showing the effects

of a change of temperature upon the duration of the embryonic period. Table XV gives data derived from transfers made between two temperatures in the range in which development can go to completion. The calculations in columns 6 and 7 are made on the as-

TABLE XIV

EFFECT OF LARVAL PERIOD AT T_1 ON THE PUPAL PERIOD AT T_2

T_1	T_2	Egg-Larval Period (Hours)		Pupal Period (Hours)	
		Male	Female	Male	Female
27.77°	27.77°	94.8±0.14	94.3±0.16	.....	80.0 ±0.11
22.00°	27.77°	141.7±0.13	149.9±0.16	84.2 ±0.12	79.8 ±0.09
27.77°	22.00°	94.4±0.13	93.5±0.12	128.4 ±0.16	120.2 ±0.11
22.00°	22.00°	141.6±0.21	149.9±0.17	126.5 ±0.13	121.4 ±0.14
24.93°	24.57°	113.3±0.14	113.6±0.16	100.51±0.08	96.78±0.07
18.06°	24.57°	241.3±0.32	259.1±0.32	103.95±0.06	99.33±0.07
24.93°	18.06°	112.2±0.13	113.4±0.14	198.4 ±0.12	190.2 ±0.10
18.06°	18.04°	243.2±0.53	262.4±0.50	204.4 ±0.15	197.0 ±0.14

TABLE XV

EMBRYONIC PERIOD—PARTIAL DEVELOPMENT AT T_1; REMAINDER AT T_2

T_1	T_2	Total Duration (Hours)	Hours at T_1	Hours at T_2	Calc. Hours at T_2	Calc. T_2 − Obs. T_2	Percentage of Difference
29.00°	16.16°	49.79±0.11	4.25	43.54	42.99	−0.55	− 1.26
29.00°	16.16°	27.99±0.080	12.25	15.74	16.40	+0.66	+ 4.19
29.00°	.....	17.18±0.033	17.18	.....	.....	.....	.....
16.16°	29.00°	28.12±0.058	14.00	14.12	12.97	−1.15	− 8.14
16.16°	29.00°	47.59±0.054	42.17	5.42	4.50	−0.92	−17.00
16.16°	.....	57.13±0.17	57.13	.....	.....	.....	.....

sumption that proportionally equal periods at different temperatures represent equal degrees of development. The values in the last column are based on the actual durations at the second temperature (col. 5).

Data from experiments in which the second temperature is one which the entire embryonic period will not survive are shown in Table XVI. Here the last column gives the time spent at T_1 (in the

range of normal development) before being placed at T_2 (lethal to complete development) for the period given in column 5, after which the eggs are replaced at T_1. The "calculated total time at T_2" represents the calculated duration of the embryonic period if it could proceed to completion at T_2 at the same rate as it did for the short

TABLE XVI

EMBRYONIC PERIOD. DEVELOPMENT AT TWO TEMPERATURES AT ONE OF WHICH, T_2, THE ENTIRE EGG PERIOD CANNOT BE COMPLETED

T_1	T_2	Total Duration (Hours)	Hours at T_1	Hours at T_2	Calc. Total Time at T_2	Hours at T_1 before Shift to T_2
24.81°	.....	21.13±0.017	21.13	.....	.....	.....
24.81°	33.1°	21.21±0.043	16.21	5.00	21.5	0.25
24.81°	33.1°	21.20±0.033	17.95	3.25	21.6	5.25
24.81°	33.1°	21.46±0.028	17.71	3.75	23.2	8.50
24.81°	33.1°	20.91±0.025	16.91	4.00	20.0	12.25
24.81°	33.1°	20.90±0.030	16.25	4.65	20.1	16.25
24.81°	14.00°	33.06±0.028	17.56	15.50	91.7	0.25
24.81°	14.00°	32.63±0.031	17.63	15.00	90.6	6.25
24.81°	14.00°	33.28±0.025	17.28	16.00	87.8	8.25
24.81°	14.00°	32.63±0.027	17.63	15.00	90.6	11.75
24.81°	14.00°	34.6 ±0.09	16.25	18.35	79.7	16.25
24.81°	10.3°	55.3 ±0.29	21.3	34.0	.....	0.25
24.81°	10.3°	44.2 ±0.04	19.2	25.0	275	9.25
28.96°	.....	17.26±0.031	17.26	.....	.....	.....
28.96°	34.80°	Of about 150 eggs, only 1 hatched				0.25
28.96°	34.80°	17.26±0.025	16.26	1.00	17.3	5.75
28.96°	34.80°	17.58±0.037	16.58	1.00	25.4	9.75

period passed at this temperature. This is determined by making the same assumptions as for obtaining the calculated values for Table XV.

DISCUSSION

GENERAL OBSERVATIONS ON DATA

Except for care in obtaining them, and for the accuracy of the methods employed, the foregoing data (Tables VIII, IX, and X, and Figs. 1, 2, and 3) are not different from other complete data of the same kind (e.g., Ludwig, 1928; Ludwig and Cable, 1933; Bliss,

1926). Thus, the duration-temperature curves show a minimum point with rapid but short rise above this temperature at the upper temperature limit, and a slower, but more extensive, increase in duration at successively lower temperatures. This seems to be typical of all biological reactions.

A comparison of the curves will show the minimum point to occur at different temperatures for the three stages. If the time axes are adjusted so that the three curves can be superimposed, it is observed that the larval period (Fig. 2) deviates from the embryonic and pupal periods (Figs. 1 and 3) more than these do from each other. This accounts for the relative constancy of the ratios $\frac{\text{embryonic period}}{\text{pupal period}}$ at different temperatures (Table XI), while the ratios involving the larval period show trends away from the average at the extremes of the range. From this, and because of the way in which the larval period enters into the ratios, the values for $\frac{\text{embryonic period}}{\text{larval period}}$ vary in the opposite direction from the ratio $\frac{\text{larval period}}{\text{pupal period}}$. On the basis of these data alone, the assumption may seem justified that the effect of temperature on the larval period is in part an effect upon the substratum (i.e., the live yeast and contaminating bacteria). However, a comparison with Figure 6 of Ludwig's 1928 paper (p. 370) and with the calculations from the data of Peairs (1927; and Table XVII in this paper) indicates that this is not the case: that there may be an effect of the temperature upon the substratum, but that the shape of the larval period duration-temperature curve is also dependent upon developmental (genetic) factors. Another point in favor of this last interpretation is the fact that genetically different lines of *Drosophila* have duration minima and upper and lower temperature extremes at different values (cf. data from Plunkett, 1926; Krafka, 1920; Child, 1935; Margolis, 1935; and the differences between males and females in my own data, Table IX, last column, and Fig. 2). The variability of interstage ratios with temperature may therefore be considered the rule, the constancy in the present ratios, $\frac{\text{embryonic period,}}{\text{pupal period}}$ being exceptional and probably accidental.

The sex differences in the larval and pupal periods are interesting. They may be interpreted as meaning, in the larval period, that at least some of the genes which determine sex also affect the duration of development, and that the actions of these genes are differently

TABLE XVII

INTERSTAGE RATIOS AT DIFFERENT TEMPERATURES FOR A NUMBER OF INSECTS
Calculated from the data of Peairs

Temp. °C.	*Lucilia caesar* Larva / Pupa	*Calliphora vomitoria* Larva / Pupa	*Musca domestica* Larva / Pupa	*Sarcophaga carnaria* Larva / Pupa
40	.....	.....	.....	0.79
35	0.88	0.74	1.13	0.80
30	0.86	0.76	1.14	0.74
25	0.82	0.77	1.22	0.85
20	0.80	0.79	1.16	0.89
15	0.83	0.69	0.99	0.88
10	0.70	0.58	0.95	0.92
8	0.50	0.36	0.71	.....

Temp. °C.	*Chloridea obsoleta* Larva / Pupa	*Pontia rapae* Larva / Pupa	*Leptinotarsa 10-lineata* Egg / Larva	Larva / Pupa	Egg / Pupa
35	0.64	.....	.....	.....	.....
30	0.70	1.20	.....	1.98	.....
25	.....	1.50	0.35	1.88	0.66
20	0.65	1.35	0.39	1.74	0.69
15	.....	1.39	0.44	1.45	0.64
10	.....	1.12	0.45	0.93	0.42

affected by temperature than are all other genes controlling development as a whole; or that genes affecting duration of development, independently of sex, are to be found in the X-chromosome, and that some of these behave like eosin in that two doses of these genes are more effective than one. Probably both mechanisms are true.

Since the difference between male and female pupae remains proportional to the duration of the pupal period, it would seem that the factors causing this sex difference were probably active before the

onset of this stage. This is supported by the fact that such important sex organs as the ovaries and testes have already been laid down in the larval period.

Table XIV provides evidence to show that simultaneously occurring processes need not have exactly the same temperature coefficient throughout the entire temperature range of the organism. Thus, in the range 22°–28° the processes determining puparium formation (and hence determining the beginning of the pupal period) proceed at the same rate as the processes involved in development as a whole. As a consequence, at both 22° and 28° the puparium is formed at the same point relative to other developmental processes; the duration of the pupal period is unaffected, therefore, by the

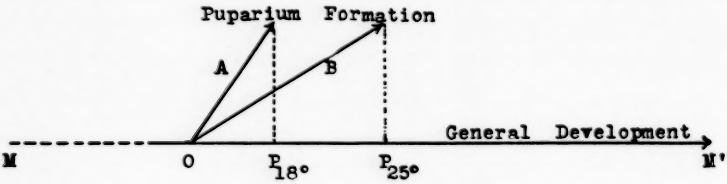

FIG. 3*A*.—See text for explanation

temperature at which the larval period occurred (compare last two columns, rows 1 and 2, and again 3 and 4, Table XIV).

In the range 18°–25° the two sets of processes have different temperature coefficients, the relative rates of these processes being different at 18° from what they are at 25°. This is made clear from Figure 3*A*. The distance MM' represents the course of general development (development as a whole). The line A (and B at another temperature) represents the course and duration of those processes which determine puparium formation independently of general development. If the temperature coefficient of the latter processes is less than that of general development, then, at a higher temperature A will occupy a relatively longer period (as represented by B) than at a lower temperature. Further, if the distances MO and OM' are adjusted so that they coincide for both 18° and 25°, the larval period at 18° is represented by MP_{18}, and at 25° by MP_{25}, while the pupal periods are represented by $P_{18}M'$ at 18° and by $P_{25}M'$ at 25°. This means that if the larval period is passed at 18° the pupal period will

be longer at either 18° or 25° than if the larval period is passed at 25° (i.e., $P_{18}M' > P_{25}M'$; cf. values in last two columns of Table XIV for rows 5 and 6, and again for rows 7 and 8).

The data in Table XVI are particularly important in that they show development to proceed to a measurable extent at temperatures at which complete development cannot take place. In column 6 the calculated values for "entire development" at the lethal temperature are of such order that they would fall very close to the curve extrapolated from the values found in the normal range (Fig. 1).

When the calculated values in column 6, Table XVI, are compared with each other, comparing only those involved in changes between the same two temperatures (as 25°–33° or 25°–14°), it is observed that they differ from each other, their values depending, apparently, upon which portion of the egg period is spent at the extreme temperature. This is particularly noticeable in the 34.80° transfers, in which an exposure at the beginning of development kills the eggs, an exposure after about one-third of embryonic development yields a calculated duration of 17.3 hours at 34.80°, and an exposure at the middle of the embryonic period a calculated duration of 25.4 hours. This simply means that the temperature coefficient of development is different in different parts of the same stage. The "percentage differences" in the last column of Table XV and the work of Ludwig and Cable (1933) support this interpretation. Brown (1927) came to what is essentially the same conclusion on the basis of Bliss's and others' work; hence this phenomenon is not uncommon.

Table XV provides data which can be interpreted in the same way. Though the data are inconclusive, they seem to indicate that the course of embryonic development is different at different parts of the stage; i.e., at one temperature the relative durations of successive periods is different from the durations at another temperature. This means, as before, that successive periods within the same stage have different temperature coefficients.

ANALYSIS OF TEMPERATURE EFFECTS

Three hypotheses, of those advanced, have been generally considered to describe the relation between durations or rates of bio-

logical processes and temperature: (1) Van't Hoff's (1884), (2) Krogh's (1914), and (3) Arrhenius' (1915) hypotheses. The first two will be examined together, the third by itself; and it will be shown that none of these can be used to explain all of the observed results.

1 and 2. When the preceding data are presented as $1/t$ (t = duration) at different temperatures (Figs. 4, 5, and 6), it becomes

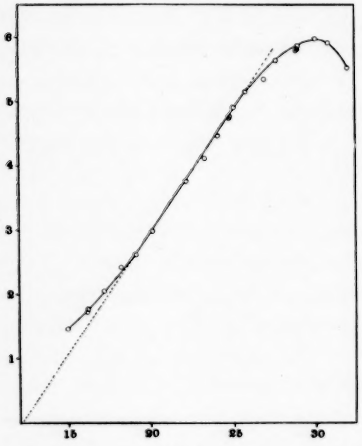

FIG. 4

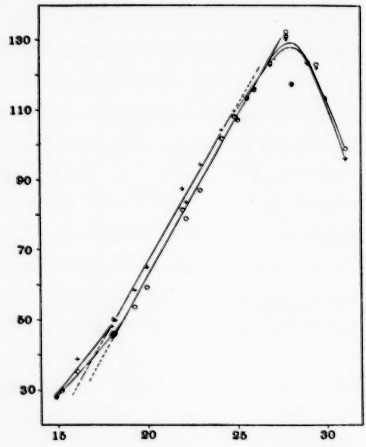

FIG. 5

FIG. 4.—Effect of temperature on the 'rate" of embryonic development. Abscissa, temperature; ordinate, 1/duration$\times 10^2$.

FIG. 5.—Effect of temperature on the "rate" of larval development. Abscissa, temperature; ordinate, $1/t \times 10^4$. + = male values; ○ = female values.

obvious that Van't Hoff's $Q_{10} = \frac{v_{T+10}}{v_T}$ does not remain constant. Table XVIII illustrates this point, the values being taken from the smooth line drawn through the points.

This falling-off of Q_{10} with increasing temperature is to be found in all other data of this type, and to a slight extent even in simple chemical reactions. Some other examples from development data are the results of Krafka (1920), Krogh (1914), Loeb and Northrop (1917), Sanderson and Peairs (1914), and others.

Since the Q_{10} of Van't Hoff has no theoretical significance, and

especially since it does not remain constant, it has practically no usefulness for theoretical purposes. For these reasons Krafka, Krogh, and others have suggested that its use be dropped altogether —a suggestion which has not yet been widely followed, though it should be.

It was just such variation in Q_{10} which led Krogh, and independently Sanderson and Peairs, to postulate the relation

$$v_{T+10}=v_T+K_{10}\,,$$

a relation which is supposed to hold over a longer range than Van't Hoff's. This equation means that the velocity of the process under consideration is a linear function of temperature, and is better expressed in the usual form

$$v=kT+c\,.$$

For many physiological processes this relation holds true, but apparently only when few measurements far apart in the temperature range have been made. It is useful, therefore, to biologists (particularly field workers) who wish to have a rough idea of the duration of various developmental stages at different times of the year. Where careful measurements at a large number of points have been made, particularly at temperature extremes, it is found that the curve deviates markedly from linearity (see Figs. 4, 5, and 6).

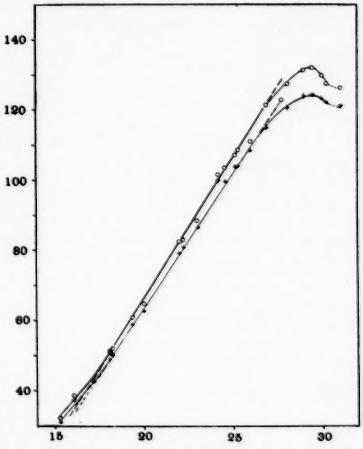

FIG. 6.—Effect of temperature on the "rate" of pupal development. Abscissa, temperature; ordinate, $1/t\times 10^4$. + = male values; ○ = female values.

This departure from linearity at extremes was observed by Krogh in his own data, and by practically every other worker in this field. In fact, every case examined, which covers the entire temperature range of the particular process, and with measurements at close temperature intervals, shows that the relation between velocity of the

process (or $1/t$) and temperature is sigmoid. Important in the present connection are the data of Loeb and Northrop (upper end of the range), Krogh, Krafka, Ludwig, and others. In every case a sigmoid curve would better fit the facts than the straight line drawn by the authors. The only apparent exceptions, where large amounts of data are available, are the results of Peairs; and here the evidence from viability indicates that the entire temperature range has not been covered.

From the "straight" lines drawn through the experimental points certain important conclusions have been drawn in the past. The in-

TABLE XVIII

Q_{10} CALCULATED FROM CURVE VALUES

Temp. Range (°C.)	Q_{10}				
	Egg	Larva		Pupa	
		Male	Female	Male	Female
15–25	3.29	3.75	3.74	3.37	3.38
18–28	2.47	2.63	3.22	2.49	2.52
20–30	1.98	1.76	1.89	1.95	1.98
28–31	1.09	0.15	0.16	1.00	0.94

NOTE.—Reference to the curves shows that the values for Q_{10} between 28° and 31° are too large, especially in the egg and pupal periods.

tercept (c in the preceding equation) upon the T-axis has been considered as a "developmental zero," or "physiological zero" (also, "threshold of development"). This is defined as meaning that development ceases as soon as the organism is cooled to or below this point. That this is contrary to the facts is to be seen from several sources of evidence. First, the sigmoid shape of the actual rate-temperature curve indicates that the rate of development is greater than zero at the point at which the extrapolated "straight line" crosses the T-axis, i.e., zero rate is approached asymptotically. In most cases this can only be observed by drawing the curve through the experimental points at the lower temperatures, since development of the entire stage usually ceases at a temperature above the

"developmental zero." Secondly, if exposure to temperatures slightly below "developmental zero" for a short enough interval is permitted, some development always takes place. Thus, in the present experiments no eggs hatched below 14°; the best straight line drawn (by eye) through the points (Fig. 4) intercepts the abscissa at about 12°; yet, when eggs in the middle of the embryonic period were placed for 25 hours at 10.3° and then brought back to 24.81°, they hatched after a total of 44.2±0.035 hours. This gave a total time spent at 24.81° of 9.1 per cent less than expected (21.13±0.017 hours) if no development had occurred at 10.3°. In other words, 9.1 per cent of embryonic development took place in eggs kept for 25 hours at 10.3°, nearly 2° below "developmental zero." If development went to completion at this temperature, a total egg period of 275 hours would be expected. It must be concluded, therefore, that some development does take place at temperatures below the "developmental zero"; hence, that "developmental zero" as above defined is not an actual physiological entity.

Other evidence of the foregoing type exists. Thus, Parker (1930) found no hatching of the eggs of *Camnula pellucida* or of *Melanoplus mexicanus mexicanus* below 17°, which he therefore assumed to be the "developmental zero" for these two species. When eggs were kept at 17° below this point (i.e., at 0° C.) and then brought to a temperature at which development proceeds to completion, it was found that they hatched in a shorter time at the higher temperature than if the eggs had not been cooled. The longer they were exposed to the low temperature, the sooner they hatched when warmed to a temperature above 17°, so that after 500 days at 0° C. eggs hatched in from one-fifth to one-third the normal time (without low temperature exposure) at 22°–37°. This could only mean that various embryonic processes were proceeding during the exposure to 0°, although hatching does not take place at this temperature. In other experiments in which eggs were placed for alternate periods at temperatures above and below 17°, the total duration of development was less than that expected on the assumption that development takes place only above "developmental zero." The fact that these eggs are diapause eggs does not in any way invalidate the reasoning, the exposure to low temperatures being more than enough to overcome

the diapause effect. Similar observations were made on the nymphal stages of *M. mexicanus mexicanus*. Other data of the same kind are to be found in the papers of Ludwig, and Ludwig and Cable.

There seems to be some evidence contrary to the foregoing. For example, Ludwig (1930) found that exposure of eggs of the Japanese beetle to temperatures below those at which they can hatch resulted in these eggs taking longer to develop at the normal temperature range than did eggs not so treated. This is not an isolated instance, but is fairly common; Ludwig refers to others. Such results may be interpreted in the following way: Some developmental processes probably always occur at temperatures at which the organism is not killed by the cold. This is borne out by the previously cited experiments below "developmental zero." Some of these processes may be slowed up more at low temperatures than others are. After a period this must cause "disorganization" of development to such an extent that some time must elapse, when the organism is raised to a higher temperature, before the processes become sufficiently correlated for development to proceed normally again. (If the "disorganization" has gone too far, the organism cannot complete development.) In this way the total duration of the stage at the temperature at which it completes development is prolonged. An example is to be found in the present data (lines 12 and 13, Table XVI). Eggs exposed to 10.3° never hatch at this temperature, but will hatch if brought to a higher temperature provided they have not been kept at 10.3° too long. As previously pointed out, a 25-hour exposure at 10.3° shortens the necessary exposure at 24.81° for hatching; it was found, however, that if the eggs were left at 10.3° for 34 hours and then placed at 24.81°, the duration of development at the latter temperature was 21.3 ± 0.29 hours and the mortality very high (about 90 per cent). This duration is not significantly longer than the normal duration at 24.81° (21.13 ± 0.017 hours) but is 11 per cent longer than the duration at 24.81° after an exposure of only 25 hours to 10.3°. Here a longer exposure to a low temperature has completely reversed the effects of a short exposure.

If the preceding interpretation is incorrect, all exposures to temperatures too low for development to go to completion should show neither a decrease in time of development when brought back to

temperatures in the range of normal development nor "harmful" effects. The author has not encountered such data in the literature. It is necessary to conclude, therefore, that no sharp "developmental zero" exists; and, furthermore, it is predicted that careful experiments with large numbers of organisms exposed for appropriate periods at low temperatures above that at which they are killed will disclose some development at these temperatures.

In the simple form in which the straight-line hypothesis of Krogh is presented, no account is taken of the falling-off of the rate at the highest temperatures at which development goes to completion. Any theory which is to explain the relation between rate of physiological processes and temperature must take this fact into account.

Blackman (1905) and Pütter (1914) have offered explanations for the inapplicability of Van't Hoff's rule and particularly for the effects at higher temperatures. Blackman showed that, if processes acting adversely increase in velocity with temperature as the process being studied does, then these adverse factors will act more and more effectively with increasing temperature. This will result in the measured process ending sooner and at a lower value at higher temperatures than may be expected from the Q_{10} at low temperatures. The rate-temperature curve may, under such conditions, have a long straight portion. Finally, at the upper end of the temperature range, the rate curve may even become concave to the T-axis. At the lower end of the curve, some other "limiting factor" may enter to stop complete development. For this factor Heilbrunn (1925) suggests protoplasmic viscosity.

Such a scheme has been put into mathematical form by Krafka (1921):

$$y = A^x + Bx^n ,$$

where y = rate, x = temperature in 10° intervals, and $A = Q_{10}$. Here Bx^n is the "depressing" factor which straightens and finally deflects downward the normally exponential curve of Van't Hoff. This formula fits Krafka's data only approximately.

The theory of Blackman, though applicable to the data on duration so far as it yields a qualitative explanation of the facts, cannot be used to explain all the data. For example, it offers no mechanism

for the variation in ratios between stages at different temperatures (Table XI), nor does it explain the facts presented in Tables XV and XVI, nor does it shed light on the effects of low temperatures.

Pütter explains the deviations from the Van't Hoff theoretical curve on the assumption that a number of reactions with different temperature coefficients are involved. If these form a chain of reactions, of which one is much slower than the others, the slowest reaction, he assumes, will determine the rate of the entire process. Under such conditions the measured temperature coefficient will decrease continuously as reactions with lower and lower temperature coefficients become successively the slowest reactions. This system was employed later by Crozier (1924) to explain the deviations from Arrhenius' equation. The inadequacy of such a scheme is discussed below.

Neither Van't Hoff's nor Krogh's hypotheses, therefore, however modified, can be made to fit development data throughout the entire temperature range, and consequently should be discarded as a means of explaining the nature of development from such data.

3. Arrhenius' theory has the advantage over the preceding in that he has given to it a theoretical derivation. The Arrhenius relation is between the velocity constant of the reaction studied and the absolute temperature:

$$\frac{d\ ln\ k}{d\ T} = \frac{\mu}{RT^2},$$

(where μ is a constant, the "critical thermal increment," and R the gas constant). For practical purposes this equation is written in the form:

$$\frac{d\ ln\ k}{d\frac{1}{T}} = \frac{\mu}{R}.$$

From this the relation between $ln\ k$ and $1/T$ is linear. Applying this equation to a number of biological reactions, he found that the equation held tolerably well; but it was chiefly important in that it indicated, as did the values of Q_{10}, that the reactions involved were chemical in nature. No more definite conclusions were arrived at un-

til Crozier made use of it in an attempt to get at the chemical nature of various biological processes. It was assumed that, if in a catenation of reactions one was slower than all the others, the slowest reaction determined the rate of the entire process, and the μ obtained for the process was the μ of this particular link. This reaction is called the "master reaction." When, over the temperature range in which the rate of the process was examined, the data could be presented only in terms of two or more straight lines (when plotted $ln\ k$ against $1/T$), it was assumed that each linear section represented the range of a single "master reaction," and that the "breaks" were the points at which reactions with lower temperature coefficients became the slowest reactions.

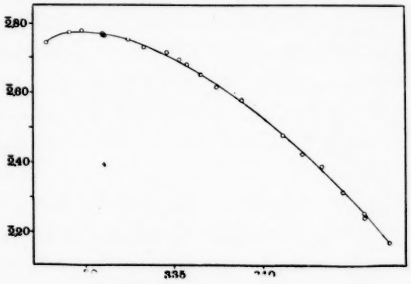

FIG. 7.—Embryonic period; the distribution of the experimental points when plotted log "rate" against the reciprocal of the absolute temperature, Abscissa, $1/T° \times 10^5$; ordinate, log $1/t$.

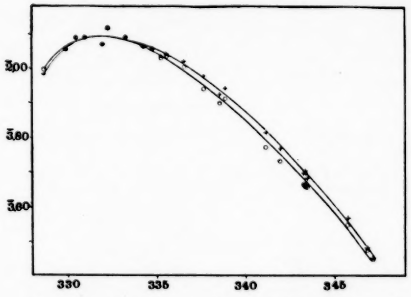

FIG. 8.—Larval period; the distribution of the experimental points when plotted log "rate" against the reciprocal of the absolute temperature. Abscissa, $1/T° \times 10^5$; ordinate, log $1/t$. + = male values; ○ = female values.

The present data, plotted as log $1/t$ (t = duration; $1/t$ is proportional to k) against $1/T$, is presented in Figures 7, 8, and 9. The distribution of points are like those of Bliss, Ludwig, and others, and resemble many of the results obtained by Crozier (1924, 1926). It is obvious that straight lines cannot be drawn to extend through the points over ranges longer than 2°. If the data from the comparatively simple processes examined by Crozier are scrutinized, it is found that, even in many of his cases,

the straight lines drawn are not justified by the facts. Instead, it is found that in many instances other lines may be drawn which may be said to fit the particular points equally well, and which intersect each other at different "critical" temperatures than those given. (See Fulmer and Buchanan, 1929, who have already criticized Crozier's work on these grounds.) In fact, it seems best to present the data as a smooth curve rather than as a series of straight lines; and this applies equally well to the data of Bliss, Ludwig, Krogh, Krafka, Loeb and Northrop, Haines (1932), and workers in this laboratory. This fact is not at all surprising when it is remembered that enzyme reactions, *in vitro* (with the possible exception of the urease reaction), do not obey the Arrhenius relation even at temperatures below that at which they are appreciably destroyed by heat (Fig. 10, data taken from von Euler, 1925).

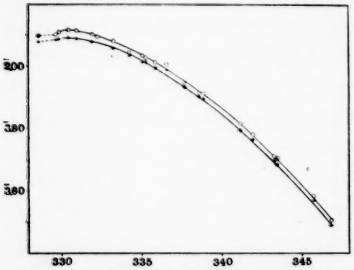

FIG. 9.—Pupal period; the distribution of the experimental points when plotted log "rate" against the reciprocal of the absolute temperature. Abscissa, $1/T° \times 10^5$; ordinate, log $1/t$. + = male values; ○ = female values.

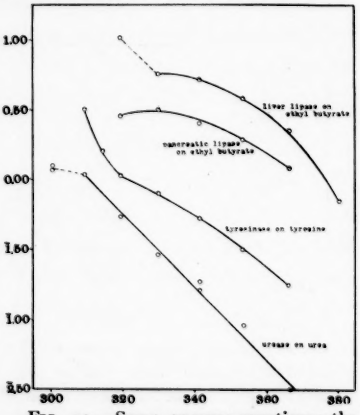

FIG. 10.—Some enzyme reactions: the distribution of the experimental points when plotted log velocity constant against the reciprocal of the absolute temperature. Abscissa, $1/T° \times 10^5$; ordinate, log k. Add 1.50 to the log k values of the tyrosinase curve. Data taken from von Euler.

In order to account for the asymmetry of some of the growth curves frequently encountered, Crozier (1926) introduced the assumption that the process underlying growth proceeds to a measurable extent as an uncatalyzed, as well as a catalyzed, reaction. This provided an additional velocity constant in Robertson's equation for growth—hence two constants to be affected by temperature. The unusual assumption must be made, how-

ever, that the catalyzed and uncatalyzed reactions proceed at nearly the same rate, and that their temperature coefficients are not much different from each other—assumptions not justified by data from reactions *in vitro*. When hypothetical values for log $1/t$ from this equation are plotted against $1/T$, a slight curvature is produced in the resultant line. This curvature can never be made sharp enough (as by adjusting the critical thermal increments of the two velocity constants) to make the theoretical curve fit the data in Figures 7, 8, and 9.

Several reasons can be advanced to show why straight lines will not be obtained. Of these, the following are perhaps the most important: first, if in a particular process there are at least two successive or concurrent reactions which are "master reactions" throughout the experimental range; and, second, if a very long series of reactions (both successive and concurrent) are involved and several of these take an appreciable time during the process (as would be expected in development).

In the first case, where two reactions are successively the "master reactions" in the experimental temperature range, the rate of attainment of the end-result is not dependent solely on k of the slowest reaction but also on the concentration of the reacting substances at each instant. Thus, in the system

$$A \overset{k_1}{\rightarrow} B \overset{k_2}{\rightarrow} C$$

(with all of A present before the reaction begins), when k_2 is many times greater than k_1,

$$\frac{dC}{dt} = k_1(A_0 - C) ,$$

so that

$$C = A_0(1 - e^{-k_1 t}) ; \qquad (1)$$

and when k_1 is many times greater than k_2 (so that the concentration of B quickly becomes equal to A_0),

$$\frac{dC}{dt} = k_2(A_0 - C) ,$$

so that

$$C = A_0(1 - e^{-k_2 t}) ; \qquad (2)$$

but when k_1 and k_2 are nearly equal, B attains a maximum value only slowly, so that it is continuously variable. Under these conditions

$$C = A_0\left[1 + \frac{k_2}{k_1 - k_2}\, e^{-k_1 t} - \frac{k_1}{k_1 - k_2}\, e^{-k_2 t}\right]. \qquad (3)$$

Assuming $k_1 = k_2$ at 20°, and critical thermal increments of 16,000 and 32,000 for these constants, respectively (these values commonly succeed each other in Crozier's data), the theoretical curve for values of log $1/t$ for a definite value of C was calculated from equation (3) and plotted against $1/T$ in Figure 11. The relation derived is a continuous, smooth curve for all values between 0° and 48°. By Crozier's hypothesis, equations (1) and (2), represented by the straight lines, should fit the calculated points; and the "break" indicated should be observed at 20°. Figure 11 proves this hypothesis to be contrary to the fact. "Breaks" in the foregoing types of curves have, therefore, no theoretical significance and result simply from forcing the data to yield straight lines for ranges however short. In fact, the conclusion is justified that, if the data plotted as above cannot be fitted with a single straight line, the Arrhenius equation is not applicable in describing the observations.

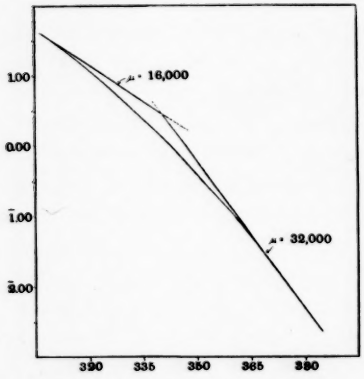

FIG. 11.—Theoretical Arrhenius curves for the reaction system $A \rightarrow B \rightarrow C$ with $\mu =$ 16,000 and 32,000 calories for the first and second reactions, respectively. The curved line represents the calculated values for log "k" throughout the region where the two velocity constants stand in the ratios 1:10 to 10:1 as they change with temperature. For ease in calculating, A_0 is taken as unit concentration. Abscissa, $1/T° \times 10^5$; ordinate, log $1/t$, where t is the time for formation of half of C.

In the case of concurrent reactions, i.e., $A \rightarrow B$ by two different paths, the most rapid reaction will impress its temperature characteristic (critical thermal increment) upon the entire process; but it

will do so only when it is much more rapid than the other reactions involved. As before, when at least two of these reactions have nearly the same temperature coefficient, the log k (or log $1/t$) $-1/T$ curve will not show a sharp break but will be a composite smooth curve asymptotic to the lines for the individual reactions. In such a case the curved portion is convex to the $1/T$ axis—a situation rarely encountered in biological processes.

Another type of system sometimes met with can be represented by

$$\begin{matrix} A \xrightarrow{k_1} B \\ C \xrightarrow{k_2} D \end{matrix} \qquad B+D \xrightarrow{k_3} E\,.$$

In such a system the time necessary for formation of a definite amount of E depends on k_1, k_2, and k_3, other things being equal. Obviously, when either k_1 or k_2 are very large, the system simplifies to

$$C \xrightarrow{k_2} D$$
$$D+B \xrightarrow{k_3} E\,,$$

and

$$A \xrightarrow{k_1} B$$
$$B+D \xrightarrow{k_3} E\,,$$

respectively, and these are equivalent to the case of consecutive reactions. A constant value for μ will be found only when two of the three constants remain many times larger than the third over the entire range of temperatures employed.

In the second case, where there are a large number of successive and concurrent reactions and where many of these occupy an appreciably large portion of the duration of the entire process, the resultant temperature coefficient must vary continuously throughout the temperature range. The more reactions of appreciable duration, and the more varied the temperature coefficients, the greater will be the curvature. Since the foregoing curves, and all other data on development which have been mentioned, show a rapidly decreasing μ with increasing temperature, it must be concluded that

the processes controlling development are quite complex and cannot be represented by such simple equations as those of Robertson[2] (1923).

In view of the preceding considerations, the Arrhenius theory is no more applicable in describing the data presented than are the theories of Van't Hoff or of Krogh.

Geneticists have already made it clear that development may not be considered as controlled by a simple master reaction. Such a fact as, for example, the action of the various genes in modifying the duration of one stage in development without markedly affecting the others runs counter to the Robertson theory. Moreover, a large number of facts have been gathered from genetics which, dealing with the differential rates and times of action of various genes, prove the presence of a large number of reactions in development. Outstanding work on this point has been done by Wheldale (1916), Goldschmidt (1927), Plunkett (1926, 1932), Krafka, and many others. (Morgan presented the general viewpoint of the geneticist in his 1926 Sedgwick Memorial Lecture at Wood's Hole.) Wright (1934) has given the most recent summary in which he points out that development is the result of a large number of chemical and physical reactions the rates and durations of which are determined by the history of the organism prior to the stage in question, correlative reactions within the organism, external environmental factors, and the actions of the genes within each cell.

Figure 12 depicts a scheme more general than the one used by Wright. This scheme has been developed in discussions with Dr. Plunkett (see Plunkett, 1932). Development is considered as being initiated at the left end and proceeding in time toward the right. The action of genes and environment is indicated only at the periphery, but actually is to be regarded as occurring throughout. Complete development is simply the sum of all the phenotypic effects. By environment ("env.") is meant all factors other than the genes. This scheme must be thought of as multidimensional and hence even more complicated than is shown above. Along with these reactions are also a number of "destructive" reactions in which certain sub-

[2] Equations, such as Pearl's, may not be criticized on this score, since they are not intended to describe any theoretical process in development.

stances produced are being removed. These last may be metabolic waste products, or substances utilized in two reactions so that they are essentially destroyed, so far as one of the reactions goes, if they are used in the second reaction.

That such a theory will fit the facts is to be expected from its general nature; but it is justified because the simple theories above out-

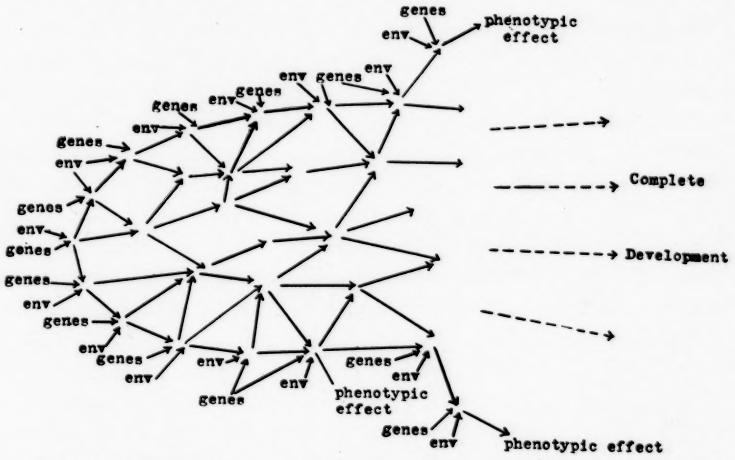

FIG. 12.—A generalized scheme to show development as a complex system of processes each initiated or modified by genetic and environmental factors. Complete development of any stage is attained when all the phenotypic effects characterizing that stage have been produced.

lined are not adequate. Moreover, all the genetic evidence points to such a complex system. It seems futile at the present stage to work out any of the steps in the body of the scheme, as the results of Schultz (1929) on the Minute reaction seem to indicate. It should be possible, however, to disentangle some of the reactions which are relatively uncomplicated; these are reactions which are located along the border of the pictured mass of reactions, or, better, are reactions which lead directly to the production of some phenotypic end-result (see Fig. 12). In all probability the processes involved in the Bar reaction are of this latter type.

Certain deductions can be made from this theory which can be tested by the facts at hand.

(1) If there are a large number of successive and concomitant reactions, no one of which controls development as a whole, the log $1/t - 1/T$ curve should show continuous curvature, as it does (Figs. 7, 8, and 9). (2) Furthermore, different stages of development may have different temperature coefficients, so that interstage ratios may vary from temperature to temperature (Tables XI and XVIII). (3) This should not only be true for entire stages, but for portions within any stage, for the stages are simply defined by certain phenotypic appearances. Column 7 in Table XV and column 6 in Table XVI can only be interpreted in this way.

(4) When many reactions proceed simultaneously and only interact at certain points, it is inconceivable that all of them will maintain proportionate rates over a long temperature range. (Table XIV and its interpretation bear out this point with regard to the simultaneous processes of general development and of pupation.) Toward both extremes of this range these reactions are likely to become more and more "disorganized," so that development becomes abnormally prolonged at these temperatures (Figs. 1, 2, and 3, and both ends of the normal Van't Hoff rate curve depressed, and hence their sigmoid shape, in Figs. 4, 5, and 6), (5) monstrosities begin to appear, and, finally, (6) development ceases to go to completion at the very extremes of the range. Just as development begins to be abnormally prolonged, differences between individuals in a given population should increase, with (7) a resultant increase in σ/mean (Table XIII). The appearance, at temperature extremes, of monstrosities, or to a lesser extent, disproportions in various structures and organs, is a common observation (e.g., in *Drosophila* the wings become abnormal and in many cases unfold only incompletely, the eyes become rough, the degree of pigmentation changes, etc.) and has been reported and even made use of by Kellicott (1916) and by Stockard (1921) in other forms.

(8) Although the organism may not be able to complete development at slightly above or below the upper or lower extremes of its temperature range, respectively, some development probably does go on for a short period before the "disorganization" of the processes results in death. This accounts for the absence of a sharp "developmental zero" and for the partial development during short exposures

to high temperatures (Table XVI). This rationale has already been employed in explaining the phenomena of short exposures to 10°.

It may be expected that environmental factors other than temperature should affect development by changing the rates of various processes. Thus, if the available food material is decreased, (9) the duration of the feeding stage (larval period) should be lengthened and (10) σ/mean should increase (Tables VII and XII*A*). Should the starvation be carried farther, a number of reactions are likely to begin to produce only minimal, and even subminimal, concentrations of substances necessary for farther development, with the result that (11) the pupal period may be prolonged and a larval-pupal correlation coefficient appear (Table XII*A*). Finally, (12) the mortality should increase and abnormalities appear. The last point is common knowledge.

(13) With regard to the action of genetic factors, a great variety of effects are possible. The present data show that the temperature coefficients for successive stages of development (omitting the embryonic period) are different in genetically different races, the males differing from the females in the larval and pupal periods. The males have a higher temperature coefficient up to about 20° than the females, the same temperature coefficient between 20° and 25°, a lower one between 25° and about 28°, and a still lower one above 28° (Figs. 2 and 5). In the pupal period the two sexes have apparently the same temperature coefficients up to 26°; above this point the females having a higher one (Fig. 6). In the light of the foregoing theory, this means that the rates of various developmental processes are modified by a change in the genotype.

Taking all the experimental evidence together, then, it is clear that the data will only fit a theory of many, more or less equally important, reactions involved in development.

SUMMARY

1. Some improved methods of handling and of timing of eggs, larvae, and pupae are presented.

2. The effects of age and of nutrition of parents upon the offspring are presented, and the means for control of these variables given.

3. It is shown that the diurnal rhythm previously reported is not encountered in all experimental work with *Drosophila*.

4. Data are presented on the durations of the egg, larval, and pupal periods when these are all spent at the same temperature and when the last is spent at a temperature different from that of the first two.

5. It is shown that at the same temperature there is little relation between the larval and pupal periods, as measured by the interstage correlation coefficient, except when the larvae have been starved (crowded), and then a large positive correlation appears.

6. The effects of exposure of the egg period successively to two temperatures in the range in which development ordinarily goes to completion, and of a short exposure to temperatures lethal to complete development are presented. In the latter case exposures at different stages in embryonic development are compared.

7. The data are discussed in relation to the theories of Van't Hoff, Krogh, and Arrhenius; and it is shown that these theories may not be applied to development, mainly because, in their application, they imply a "master" reaction, i.e., a single, controlling reaction, underlying development (with the exception of Krogh's theory, which is empirical).

8. It is shown that the data will support a theory which explains development in terms of a large number of interacting simultaneous and successive reactions.

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VARIATIONS IN SUSCEPTIBILITY TO X-RAYS OF MELANOPLUS DIFFERENTIALIS EGGS DURING DEVELOPMENT[1]

(Five figures)

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EARLY investigators of the radiosensitivity of embryos concluded that as development proceeds the resistance to X-rays steadily increases. Several recent investigations, however, have indicated that certain particular stages of insect development are exceptions to this general rule. P. S. Henshaw (1932) and P. S. with C. T. Henshaw (1933*a*) have reported that, in *Drosophila*, gastrulation is a particularly resistant stage to X-radiation capable of penetrating uniformly to all parts. These same writers have recently described (1933*b*) changes in susceptibility to alpha particles during early development, and they have found that embryos are more susceptible when the active cells are near the surface of the egg within the penetrating range of the radiation employed. Scott (1934) irradiated eggs of *Calliphora erythrocephala* and found that loss of sensitivity during development followed an irregular course which seemed to accompany changes in developmental activity. He found that during the first few hours (before definite cells were formed) the sensitivity was not as great as later, when the cellular mitotic rate was high. The sensitivity then decreased during the next few hours, but increased greatly at the time when the blastoderm was on the point of invaginating. He considered the complexity of the cell changes which must precede the invagination process as the simplest explanation of this increased sensitivity.

Although the foregoing reports agree that younger embryos are more susceptible, they indicate that this radiosensitivity is more than a matter of degree of differentiation. Dipteran embryos develop

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almost continuously; yet the small cycles in developmental activity noted are apparently associated with changes in susceptibility. Therefore, it is evident that in extensive periods of low developmental activity, changes in radiosensitivity should be even more marked. It so happens that at one stage in the development of the grasshopper embryo, *Melanoplus differentialis*, activity is very low and no visible growth occurs for a time. This inactive period (diapause) may be shortened or eliminated by experimental means. According to the preceding indications, this period should be associated with increased resistance to X-radiation; and it is the purpose of this paper to report results of such an investigation.

MATERIALS AND METHODS

The embryo (*Melanoplus differentialis*) at 28° C. grows rapidly for the first 15 days, but development then decreases considerably and by the twentieth day is in a state of apparent quiescence. This interruption of development (diapause) lasts for several weeks, after which period development is again rapid and involves much movement and visible growth. Continuous development was obtained by placing either diapause or pre-diapause eggs at a temperature of 5° C. for a month or longer before the beginning of the experiments at 28° C. The longest period of continuous activity was obtained by exposing 5-day-old pre-diapause eggs to the low temperature.

Visible changes in the embryos were noted under the binocular dissecting microscope, and occasional photomicrographs were made of the embryos fixed in 5 per cent acetic acid or in Bouin's solution.

The reader is referred to the papers by Bodine (1929, 1932*a*, 1932*b*) for material concerning factors relative to diapause, to Slifer (1931, 1932*a*, 1932*b*) for details of developmental stages, and to Evans (1934) for details concerning morphological and physiological end-points used in previous X-ray experiments.

Some indication of the developmental activity was obtained by determinations of the rate of oxygen consumption and of visible growth. The oxygen consumption rates were determined by means of a modified Krogh manometer, and the same respirometer was used for each group of irradiated and control eggs throughout the experiment.

The X-ray apparatus was the same as that employed in the previous investigation (Evans, 1934). The radiation (130 kv., 5 ma., and 35 cm. distance) was applied in one treatment, and the amount was increased by lengthening the exposure time.

RESULTS AND DISCUSSION

In a previous investigation of the effects of X-rays on the grasshopper egg (Evans, 1934), the partially developed (first-day, post-

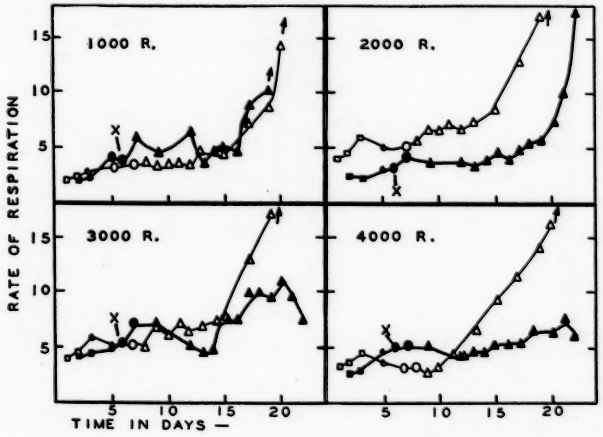

FIG. 1.—Graphs showing typical results of irradiating embryos in late blastokinesis. The rates of respiration are in terms of millimeter change of manometer fluid per hour per egg. Lighter line and open symbols refer to controls (average of 20 eggs). Heavy line and solid symbols refer to irradiated eggs (average of 20). X indicates day of irradiation. Arrow indicates hatching of first embryo.

diapause) embryo was used. This stage was obtained by placing diapause eggs at 5° C. for over a month. The material was then extremely uniform and showed visible development, as well as increased respiration, within 2 days at 28° C. The ensuing development was rapid and continuous. It was found that 1,000 *r*-units of irradiation stopped development at the time when controls were hatching, and heavier treatments resulted in a very early appearance of harmful effects.

In the present investigation similar dosages were applied to a

stage which appears from 3 to 5 days later (blastokinesis), and the differentiation and activity are more pronounced. Typical results are shown in Figure 1. The eggs with embryos in blastokinesis were much more resistant to light dosages than first-day post-diapause ones, but with heavy dosages the difference was very slight.

The next experiment was devised to make the period of development before hatching longer than that of the (first-day) post-diapause stage. Pre-diapause eggs were exposed to low temperature

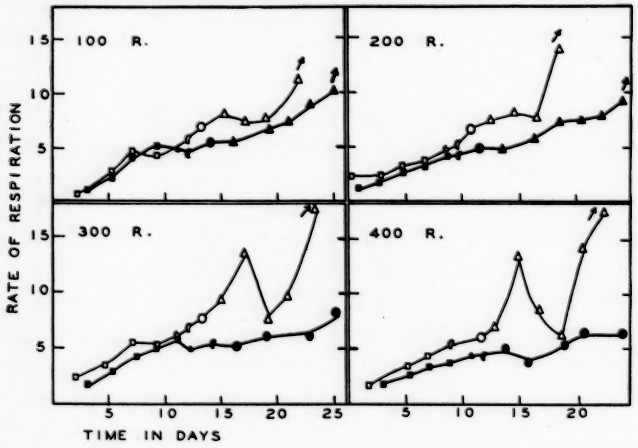

FIG. 2.—Graphs showing typical results of irradiating very early embryos (in 6-day stage). The heavy line refers to experimentals (average of 25), and the lighter curve refers to the controls (average of 25 eggs). Arrow indicates hatching of first individual.

(5° C.) until they became of the non-diapause type; and when they were placed at 28° C., they failed to stop developing when the morphological stage associated with diapause was reached. This gave a continuous developmental time of from 25 to 30 days. It was soon evident that these early embryos were extremely susceptible to X-rays. A dosage of 1,000 *r*-units, for example, resulted in almost immediate cessation of development, while this dosage administered at a later stage (post-diapause) did not stop development until after a period of over 20 days. Even dosages as low as 300 and 400 *r*-units stopped growth and further respiration after 10 or 15 days. Typical results are shown in Figure 2.

The results of the experiments on continuous development indicate that resistance increased greatly as development proceeded.

Similar experiments were now applied to discontinuous development. This type resulted when eggs were allowed to remain at 28° C. continuously. By the time the eggs were 16 days old the embryos ceased growing and remained inactive for a period of time. The very early embryos responded to irradiation as did the non-diapause organisms of corresponding ages; but when they were approaching diapause, the action of the X-ray injury was apparently delayed. Figure 3 shows, schematically, results of irradiating embryos of different ages before diapause. It is apparent from this experiment that the rapid appearance of harmful effects of X-rays was associated in some way with the rate of development.

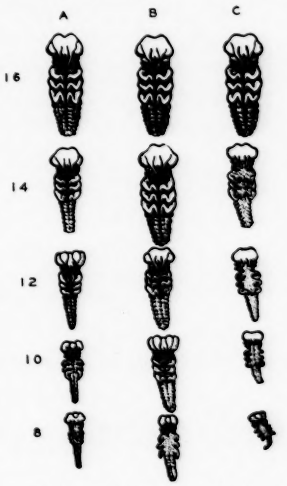

FIG. 3.—Schematic drawings made from photomicrographs and from preserved specimens showing visible changes in embryos of different ages treated with 1,000 *r*-units of X-rays. Column *A* shows the appearance of embryos at times of irradiation. The age (at 28° C.) of these embryos is indicated to the left of each. Column *B* shows the appearance of typical embryos 1 week following the irradiation. Column *C* is of embryos 2 weeks after the irradiation, showing the degree of injury.

The radiosensitivity of the diapause stage was studied next. Figure 4 shows typical results of irradiating such eggs. These particular eggs had been in diapause for 20 days when rayed, and most of them did not begin development until some 15 days later. These eggs (controls as well as experimentals) did not begin development at the same time; so the morphological condition of the majority of each lot was used for purposes of comparison. It will be noted that some eggs hatched about 35 days after they had been rayed with 1,000 *r*-units of X-rays, and it will be remembered that this dosage was always fatal to post-diapause (first-day) eggs. It will also be noted that embryos irradiated with 4,000 *r*-units reached the stage of complete engulfment of yolk before growth was stopped, whereas such a dosage given to post-diapause eggs killed the embryos before the

yolk had been completely engulfed. Although the diapause stage is morphologically and potentially similar to the first-day post-diapause embryo, it is much less susceptible to X-radiation.

The experiments on discontinuous development indicate that the physiological condition of developing organisms is important and may modify the effects one would expect from the morphological appearance.

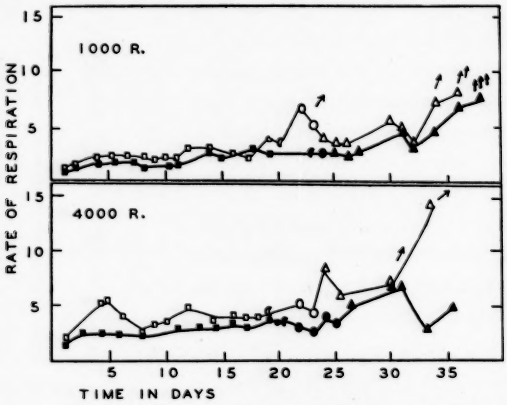

FIG. 4.—Graphs showing typical results of irradiating eggs in diapause. These eggs were shelled on the day irradiated, and the later development started in the majority of cases by the eighteenth day. Respiration is average of 15–30 eggs, and symbols indicate morphological stage of the majority of embryos. Arrow indicates hatching of individuals.

The changes in radiosensitivity during development of the grasshopper embryo may be roughly measured by comparing the latent periods following different dosages. This is done in Figure 5. The points on the curves indicate the dosages on which experimental data have been obtained. The latent period is defined as the time from irradiation to the day on which development apparently ceased as exhibited visibly or in terms of respiratory rate. Curve *1* (Fig. 5) shows that the early embryos exhibit only a short latent period and the range of dosage is rather narrow. In other words, the susceptibility of these early embryos is very high. It may be seen from Curve *2* (Fig. 5) that post-diapause embryos are more resistant and the effective dosages cover a wider range. The latent period follow-

ing an irradiation of a certain dosage (1,000 *r*-units) is ten times greater than that of the pre-diapause embryos. Curve *3* (Fig. 5) indicates that the later embryo (in blastokinesis) is still more resistant. These comparisons might be interpreted as indicating that resistance increases with development and differentiation. However, develop-

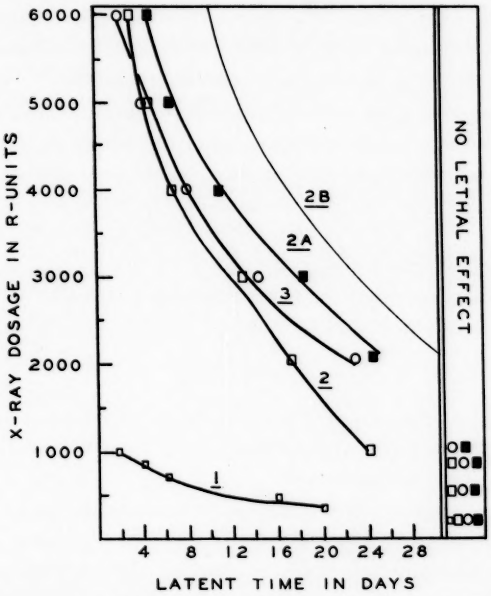

FIG. 5.—Composite graph showing the relations of developmental stages and developmental activity to radiosensitivity. Curve *1* shows the latent period following different amounts of irradiation of the 6th day pre-diapause and 6-day stage of non-diapause eggs. Curve *2* is of post-diapause embryos. Curve *3* is of still later embryos, in blastokinesis. Curve *2A* is of diapause eggs (same morphological stage as Curve *2* but usually remains inactive for two or three weeks). Curve *2A* represents the latent period counting from the time when development begins. Curve *2B* is a rough estimate of what the latent period would be if the minimum time of normal inactivity were added.

mental activity during and following the irradiation is also a factor. This will be apparent by noting the high resistance of diapause eggs as indicated in Curve *2A* (Fig. 5). These diapause eggs do not differ visibly from the post-diapause ones because the latter are obtained

by exposing the diapause eggs to low temperature for a long period of time during which the morphology does not change. Thus the chief difference seems to be that diapause embryos remain inactive, whereas the post-diapause ones begin development immediately following irradiation.

The resistance of the diapause stage of grasshopper development is similar to that of the inactive period of the mud-dauber wasp (Bodine and Evans, 1934). This resistance of mud-dauber wasp larvae was shown to be related to inactivity, as recovery was noted when larvae were kept cold for several weeks following irradiation. Some preliminary experiments on grasshopper eggs (Evans, 1934) have been repeated, and they indicate that post-diapause eggs recover from X-ray injury (within limits) if they are kept inactive (by exposure to 5° C.) for several months. Similar experiments on pre-diapause eggs do not show such marked recovery; so it appears that the original change resulting from irradiation is of a more permanent nature than that of the later stages.

CONCLUSIONS

1. Eggs of *Melanoplus differentialis* have been irradiated in different stages of development, and their subsequent respiration and development have been observed.

2. When development is continuous, the resistance to X-rays increases as development proceeds.

3. When development is discontinuous, the inactive stage shows increased resistance to, or recovery from, X-ray injury.

4. The resistance, or recovery, of the diapause embryo is apparently associated with its inactivity during and following irradiation.

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THE ACTION OF SOFT X-RAYS ON CLADOCERA (DAPHNIA MAGNA)

(Seven figures)

G. SNIDER AND H. KERSTEN
University of Cincinnati

THE action of X-rays on a wide variety of biological forms has been studied by numerous investigators (Mavor, 1923; Muller, 1928; Stadler, 1928; Goodspeed and Clausen, 1928; Curtis, 1928; Cattell, 1931; Hussey, 1932; Obreshkove and King, 1932; Henshaw and Francis, 1933; Bodine and Evans, 1934; Evans, 1934; Strandskov, 1934), but no material offers a more convenient means of study than the cladoceran *Daphnia magna*. Its small size (2 mm., approximately), short life-history (about 8 days), transparency, and ready availability from standard culture medium (Banta, 1921) make it especially desirable for these studies. With these advantages in mind, four topics were investigated, as follows: (1) killing-time curve at 45 kv., 10 ma.; (2) differential action of X-rays on anterior and posterior halves of animal; (3) susceptibility of eggs while in mother's brood-chamber; (4) influence of X-rays on growth and development of young.

METHODS

Parthenogenetic females having the second clutch of eggs in the brood pouch were used in some types of experiments. Young (1 day old) furnished the material in other experiments.

The X-ray tube (Fig. 1) had a copper target and window consisting of 0.045 mm. of aluminum and the same thickness of cellophane (Kersten, 1934).

The most intense part of the radiation had a wave-length of 1.54 Ångstrom units. Dosages varied with type of experiment. In some experiments animals were placed 8 cm. from the focal spot of the X-ray tube; in others, $3\frac{1}{2}$ cm. distant; and in still others, 3 cm. distant. Time was measured with a stop watch. Relations between soft X-rays and neighboring radiations and probable spectral distri-

bution of soft X-ray energy are shown in Figure 2. With the higher voltages the intensities were greater and more of the harder radiation was included.

RESULTS

Killing-time at 45 kv., 10 ma.—Our criterion for determining death was a complete cessation of the heart beat within 1 minute from the time the animals were removed from the X-ray tube. Using ten adult females at each trial and placing them next to each other on moist blotters in the depression of a well slide 3 cm. from the focal spot, the results shown in Figure 3 were obtained.

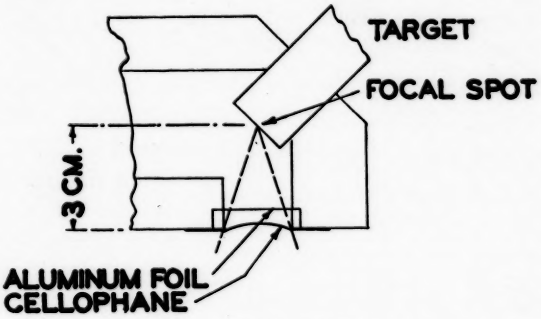

FIG. 1.—Soft X-ray tube

It will be noted that the curve descends rather steeply and uniformly from the high point of 100 per cent surviving (3 minutes) to the low of 0 per cent suriviving (9.5 minutes). At any time greater than 9.5 minutes, all animals will be dead; at any time less than this value, some or all will be living.

Differential action of X-rays on anterior and posterior halves of an animal.—The time for heart stoppage is about one-half as long when the whole animal is subjected to the rays as it is when only one-half is treated. However, the lethal effects of the rays on *Daphnia* vary according to whether the anterior or posterior half is irradiated. That is, the time required for the cessation of the heart beat is longer when the posterior half is irradiated than it is when the anterior half is exposed.

Three animals were treated at each trial—one fully exposed (con-

trol), one with the anterior half exposed, and the other with the posterior half subjected to the radiation. They were placed on a glass slide in copper rings which fitted them so snugly, they were unable to turn around, yet were moist at all times. The parts not to be irradiated were shielded with a lead screen. (See Fig. 4.)

The animals were placed $3\frac{1}{2}$ cm. from the focal spot and irradiated at 45 kv., 10 ma., for periods of time given below. The effectiveness of the rays was shown by the coloring of that part of the glass slide exposed to the radiation (Kersten and Dwight, 1933) and by the lack of coloring beneath the copper rings in which the animals were placed.

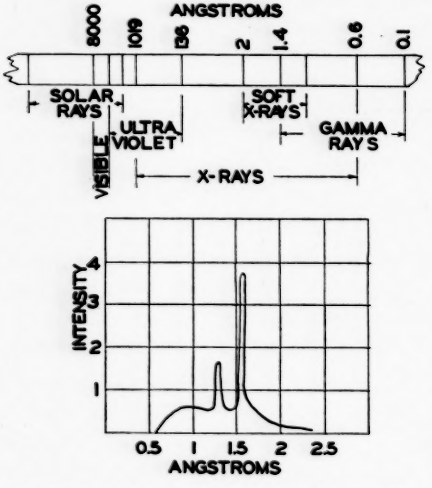

FIG. 2.—*Upper:* Position of soft X-ray region in relation to neighboring radiations. *Lower:* Probable spectral distribution of energy when tube was operated at 20 kv., 10 ma., with a copper target.

The results from eight experiments at the foregoing voltage were as follows: Animals when fully exposed to the radiation died in 9.5 minutes. Those with the anterior half exposed (including the heart) died in 15 minutes. Those with the posterior half exposed (including the heart) were still living at 16 minutes. When anterior and posterior halves were exposed (hearts shielded), both animals were living after 25 minutes. Similar results were obtained in ten experiments at 40 kv., 10 ma., $3\frac{1}{2}$ cm. distance from the focal spot of the X-ray tube.

These results are in accord with the biological principle of axial gradients (Child, 1924), namely, that the anterior end of an animal is the part undergoing more active metabolic changes than the posterior part, and is the end of the body most susceptible to adverse in-

fluences of whatever kind. Strandskov (1934) has arrived at a similar conclusion, using hard X-rays and employing flatworms as material.

Susceptibility of eggs while in mother's brood-chamber.—Irradiation of adult females for 40, 60, and 80 seconds with a dosage of 20 kv.,

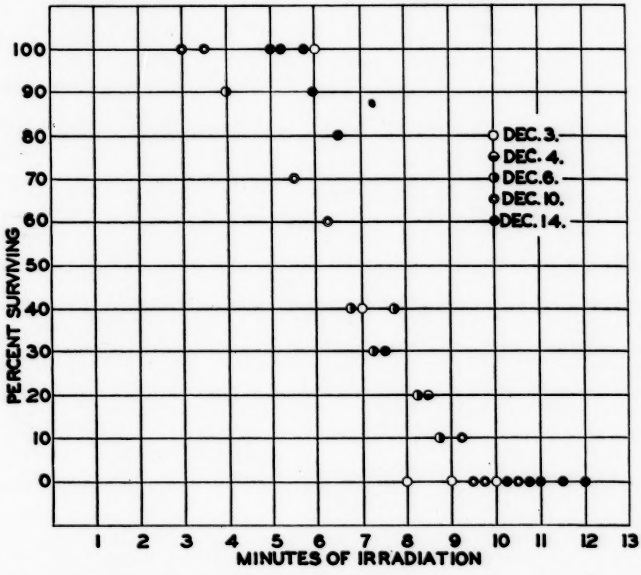

FIG. 3.—Graph showing percentage surviving as a function of the time of irradiation when the tube was operated at 45 kv., 10 ma. Note: Each point represents percentage surviving among ten animals; total animals, 310.

10 ma., 8 cm. from the focal spot of the X-ray tube caused a destruction of the eggs but no apparent effect on the ovaries (Fig. 5).

The destroyed eggs were noticed 2 days subsequent to treatment as a coalesced, dead-looking mass in the brood-chamber. In a few cases some eggs irradiated with others in the brood-chamber developed later into normal individuals. The explanation for the latter phenomenon appears to be that these eggs were in some way protected from the rays, perhaps by being farthest away from the source of irradiation or shielded by some overlying structure. It has been

observed (Henshaw and Francis, 1933) that cells undergoing mitosis are more susceptible to X-irradiation than those not undergoing this process, and this would seem to account for the apparent unharmed condition of the ovary while the eggs are destroyed.

Influence of X-rays on growth and development of young.—Eight series of experiments were performed (thirty experimental and thirty control animals in each series), subjecting animals (1 day old) to X-ray dosages of the same intensity (20 kv., 10 ma., 8 cm. from the focal spot) but varying in lengths of time of exposure, that is, 5, 25, 45, 55, and 75, seconds. Longer exposures invariably resulted in death the same or following day. These young animals were placed, six at a time, equidistantly on a moist blotter in a small Petri dish on a slowly revolving disk. Examinations were made each day after experimentation, but the most noticeable results were obtained at the end of 8 days (Fig. 6).

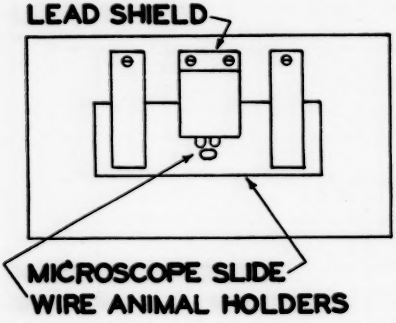

FIG. 4.—Shielding apparatus used in irradiating anterior and posterior halves of animals.

These experiments always show three outstanding results: (1) a decrease in the size of the animal which is more marked as length of exposure to the X-rays increases; (2) an increase in the inhibiting effect of the irradiation on the gonads with increased dosages; (3) a disorganized carapace and irregularity in size and direction of the spines.

The most marked effect on the gonads occurred with the 75 seconds' exposure. Animals so treated developed to adults but always exhibited an inhibition of the ovary, as shown by a decrease in the relative number of eggs laid. Those treated with lower dosages, although showing marked inhibition of ovarian growth and number of eggs laid, if given time and favorable conditions, recovered from the sterilizing influence and produced normal young.

The disorganized carapace was distinguished by its elements being

irregular in size and outline. The irregularity in spines and carapace was obviously most noticeable in 75 seconds, and least, if at all, in the exposures of 25 and 5 seconds (see Fig. 7).

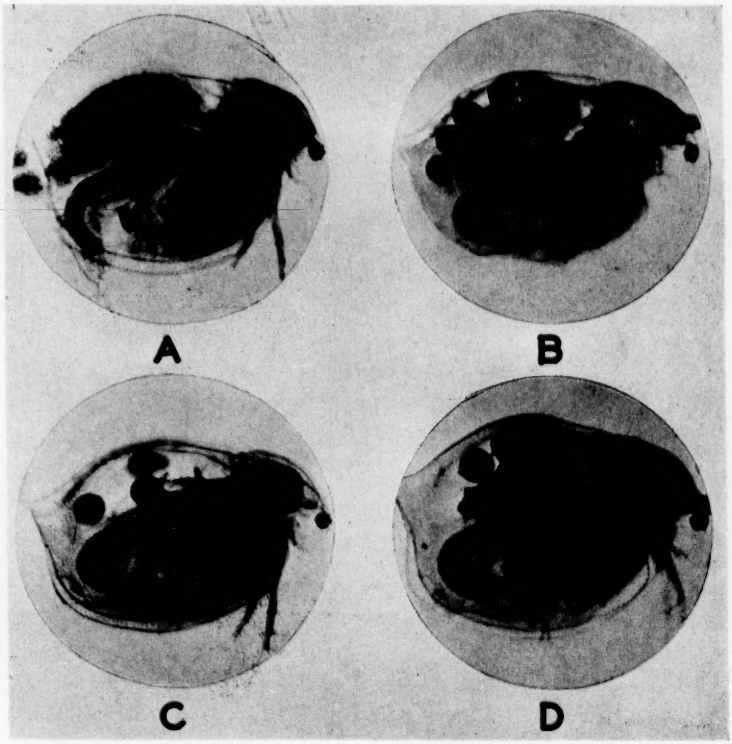

FIG. 5.—Photographs showing destruction of *Daphnia* eggs in the brood-chamber when the animals were irradiated at 20 kv., 10 ma., 8 cm. from the focal spot of the X-ray tube. *A*, control; eggs developed to embryos after 2 days. *B*, experimental; irradiated 40 seconds; eggs dead after 2 days. *C*, experimental; irradiated 60 seconds; two dead eggs and one embryo after 2 days. *D*, experimental; irradiated 60 seconds; disintegrated eggs after 2 days.

SUMMARY AND CONCLUSIONS

1. A time-killing curve (45 kv., 10 ma., 3 cm. from focal spot) has been worked out for the females in the first adult instar of the clado-

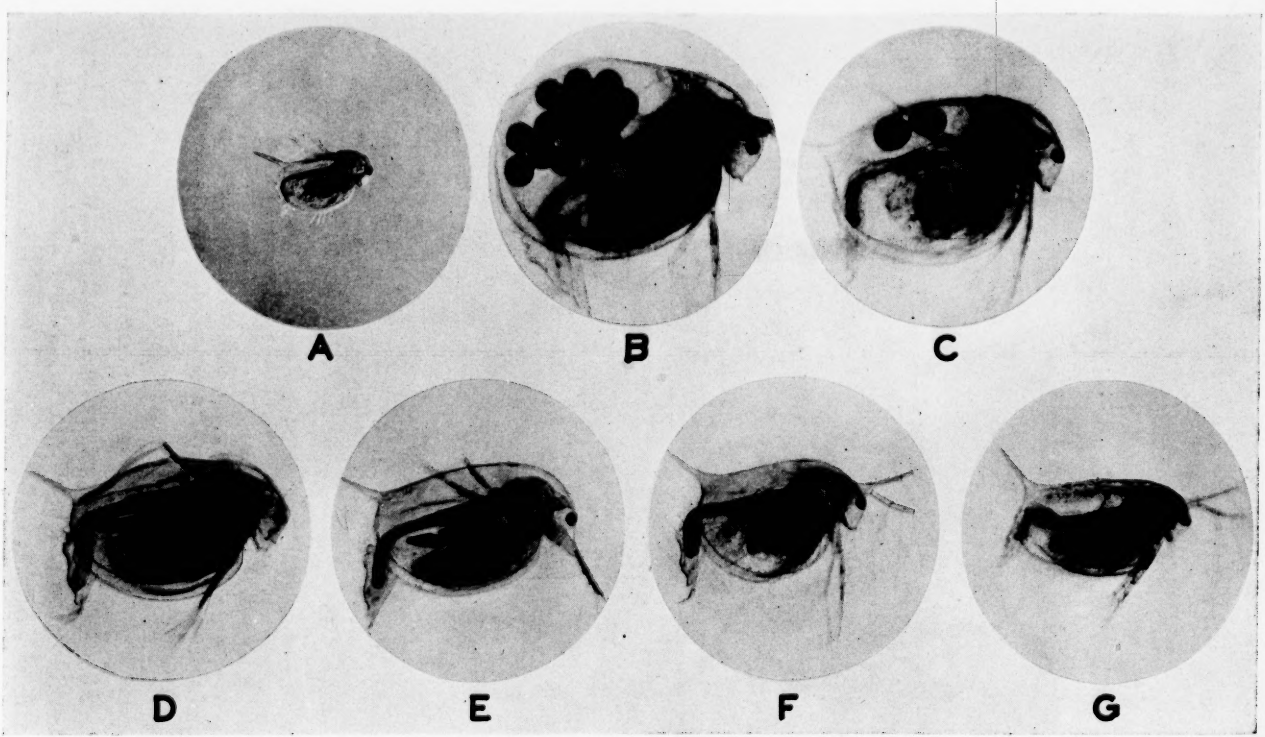

FIG. 6.—Effects of soft X-rays on the development of the water flea when the X-ray tube was operated at 20 kv., 10 ma., animals 8 cm. from the focal spot. Animals irradiated when 1 day old; photographs taken 8 days later. *A*, 1 day old. *B*, control. *C*, 5 seconds, *D*, 25 seconds. *E*, 45 seconds. *F*, 55 seconds. *G*, 75 seconds.

ceran *Daphnia magna*. The time at which all animals are dead (cessation of heart beat), above which all would be dead, and below which some or all would be living, is 9.5 minutes.

2. Differential action of soft X-rays on anterior and posterior halves of an animal has been determined wherein the same dose, lethal when administered over the same period of time to the anterior half (heart exposed), is non-lethal to the posterior half (heart

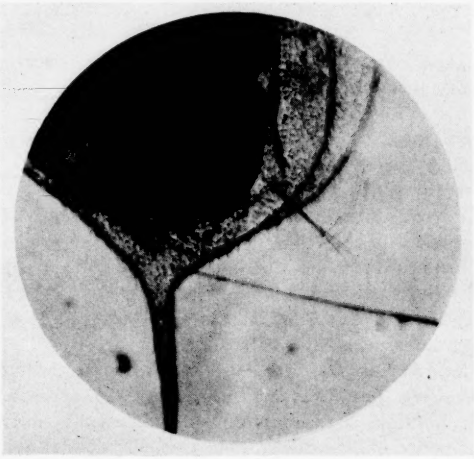

FIG. 7.—Photograph showing irregular spines on edge of carapace between caudal spines and abdominal setae.

exposed). This fact contributes to the support of Child's Law of Metabolic Gradients. When both anterior and posterior halves are simultaneously irradiated with both hearts shielded from the X-rays, the animals continue to live much longer than when hearts are exposed.

3. Newly laid eggs in the mother's brood-chamber, when treated with 20 kv., 10 ma., 8 cm. from the focal spot of the X-ray tube for 40, 60, and 80 seconds, show disintegrative changes within 2 days, although the ovary itself is unharmed. Subsequent offspring show no effects.

4. When 1-day-old animals are irradiated (40 kv., 10 ma., 8 cm.

from the focal spot) for 5, 25, 45, 55, and 75 seconds and examined at the end of 8 days, they show a distorted carapace and disorganized spines, a decrease in size progressing with increased dosage, and a sterility varying in degree with the intensity and length of time of irradiation. Up to 75 seconds' exposure the effects are transitory.

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